# Avoiding Chromatography Heartbreak: Common LC Pitfalls

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# Avoiding  Overview

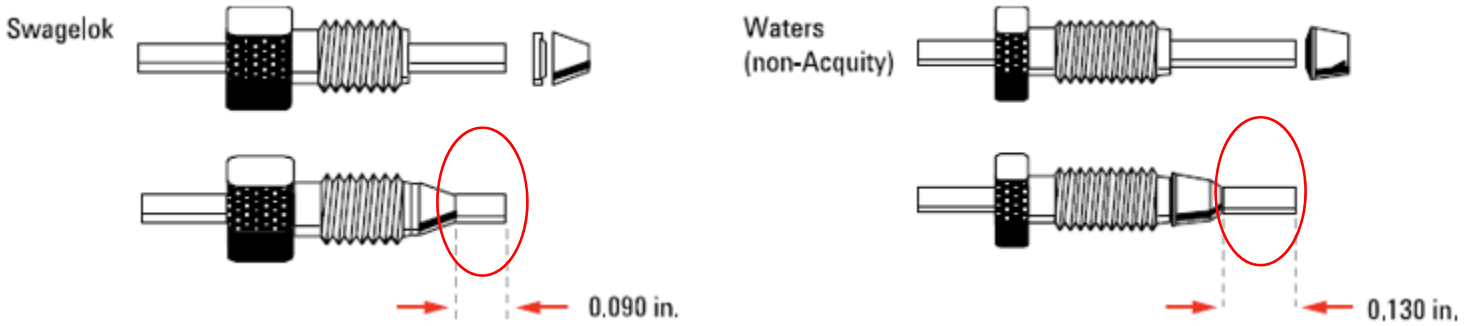
- Instrument
  - Connections
  - Performance
- Column
  - Characteristics
  - Lifetime
- Method
  - Mobile phase
  - Temperature
- Sample
  - Cleanup
  - Injection

# Instrument Pitfalls

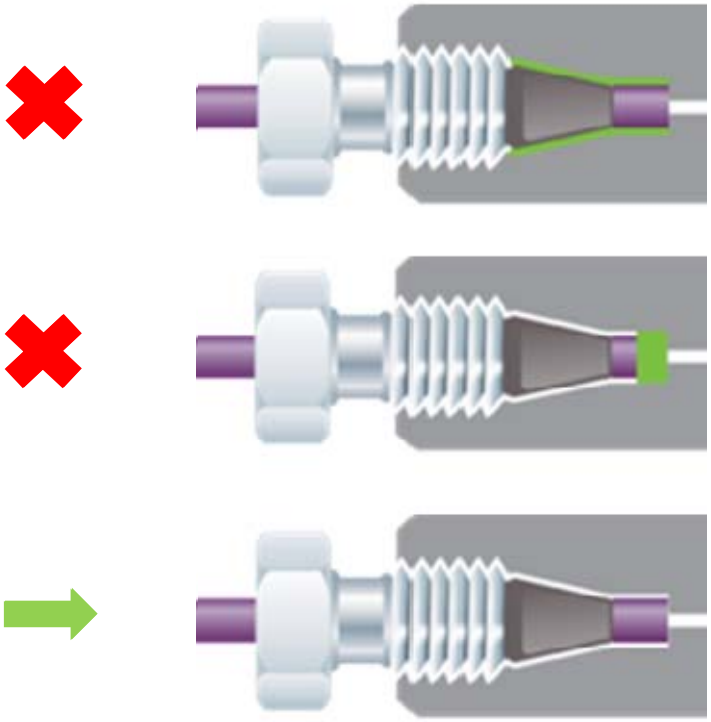
- Fittings
  - Connections
    - Improper – Results in areas where the flow does not move smoothly
- Tubing
  - ID, Length
  - No random pieces
- Detector
  - Flow cell
  - Proper settings

# LC Connections

- Problems with improper connections
  - Source of leaks
  - **Mistaken for chromatography issues**
- Making connections can vary with skill/technique
- Different manufacturers supply different types of fittings



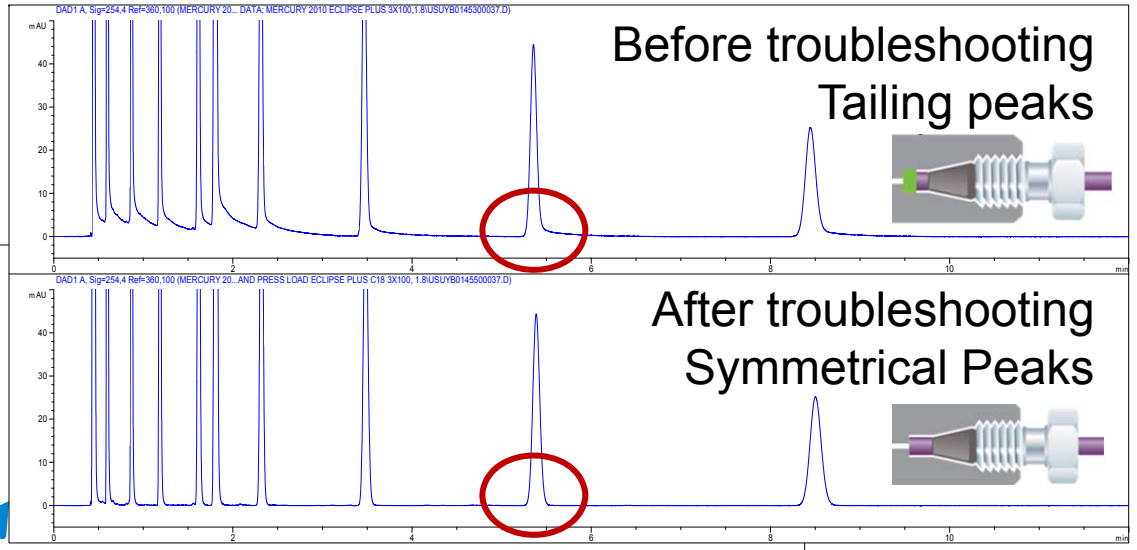
# Potential Fittings Issues

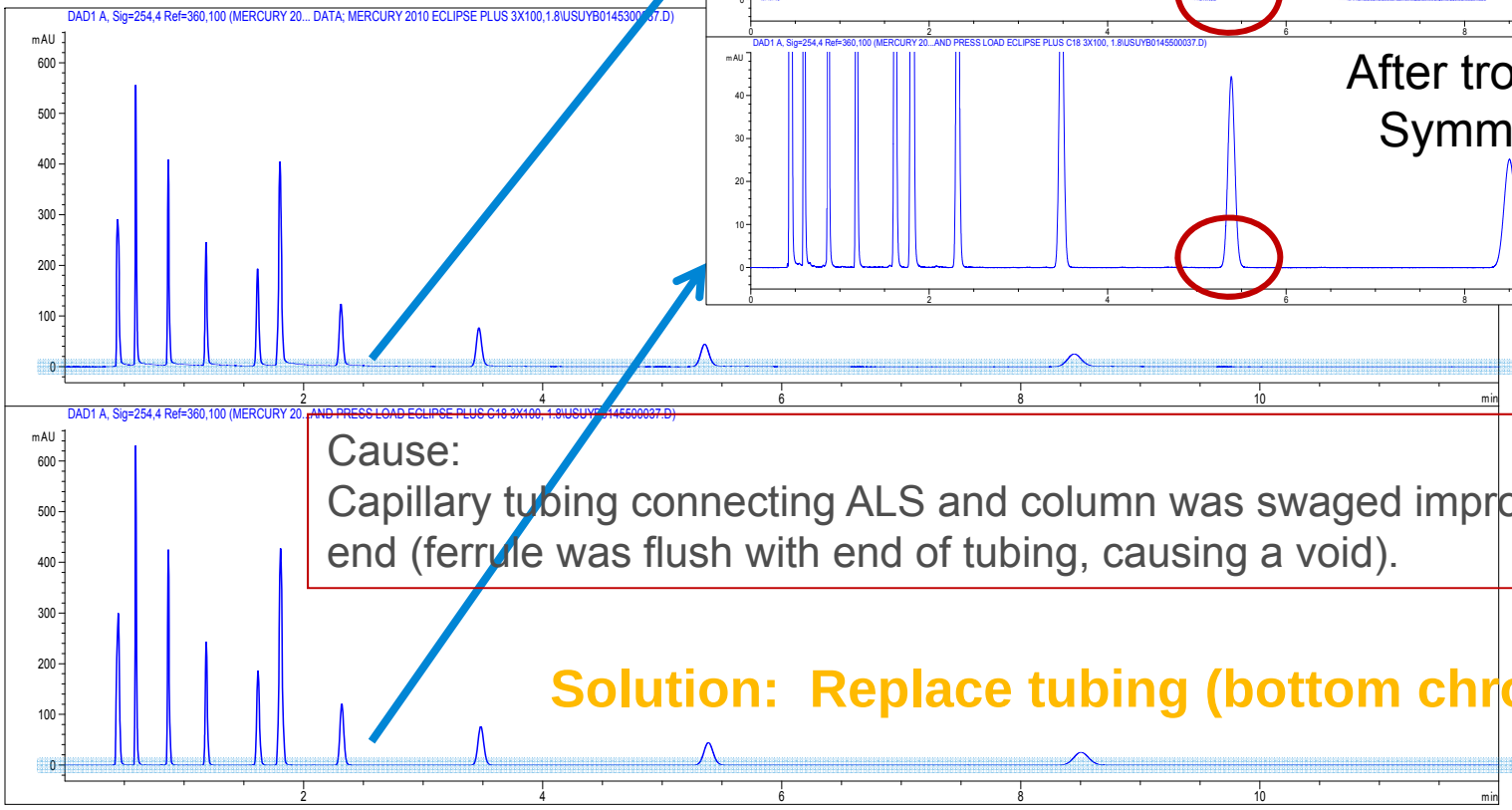


- Leak
- Peak shape problem
- No dead volume

# Poor Fitting
# Peak Tailing – Extra Column Effects

**Problem:**
**All peaks tail**
**(top chromatogram).**




Cause:
Capillary tubing connecting ALS and column was swaged improperly on the ALS end (ferrule was flush with end of tubing, causing a void).

**Solution: Replace tubing (bottom chromatogram)**

# Standard LC fittings: Swaging

## Step 1

Select a nut that is the right length for the fitting.

## Step 2

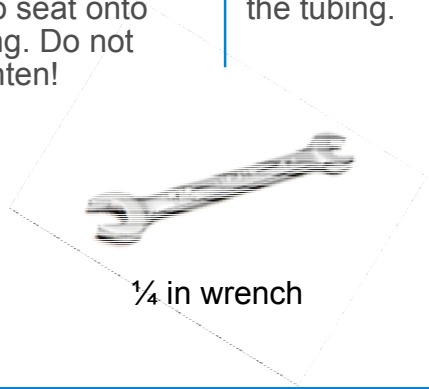
Slide the nut over the end of the tubing.

## Step 3

Carefully slide the ferrule components on after the nut. Finger-tighten the assembly while making sure the tubing is completely seated in the bottom of the fitting.

## Step 4

Use a wrench to gently tighten the fitting by 1/4 to 1/2 turn where you want to connect it; this will force the ferrule to seat onto the tubing. Do not over-tighten!



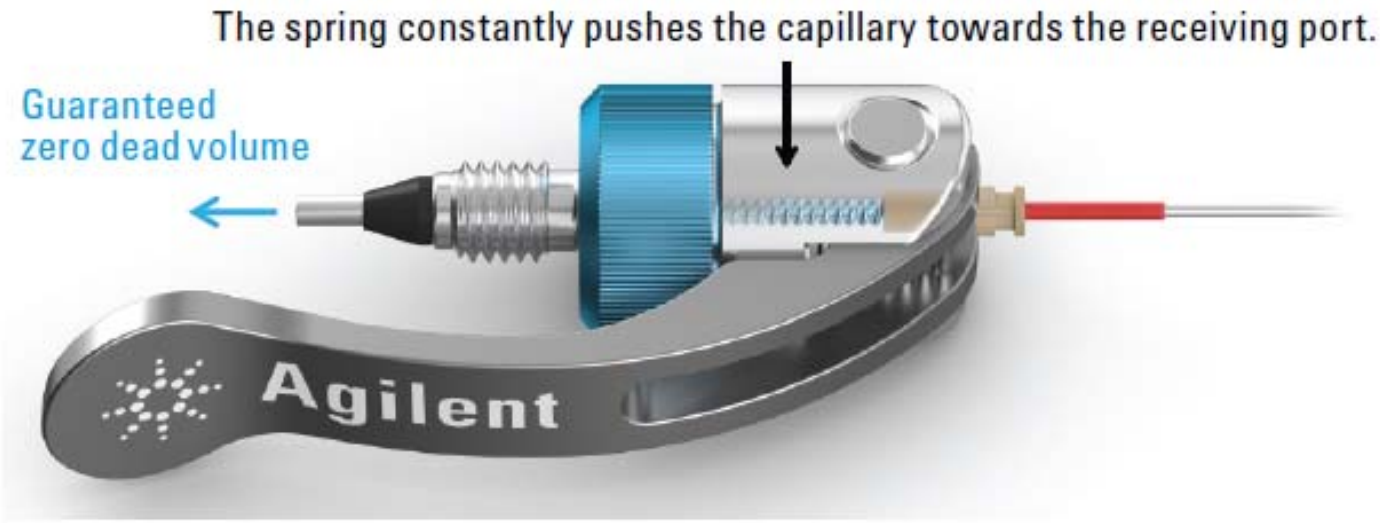
¼ in wrench

## Step 5

Once you are sure your fitting is complete, loosen the nut and inspect the ferrule for correct position on the tubing.

➢ Avoid pitfalls – Check out Agilent on YouTube
https://www.youtube.com/user/agilent

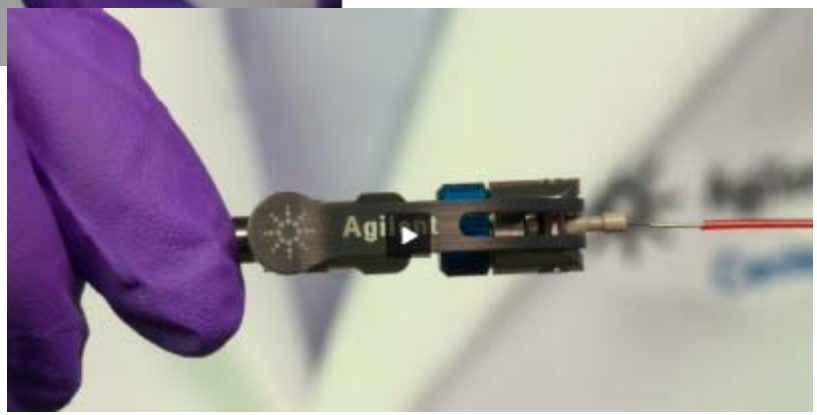
# InfinityLab (A-line) Quick Connect Fitting: Unique spring-loaded design




- Unique Spring-loaded design applies a constant force to eliminate dead volume
- Fingertight to 1300 bar

# Quick Connect: Leak-free connections with the push of a lever

Finger tighten the fitting until you feel the resistance, then close the lever



- Avoid connection pitfalls - http://www.agilent.com/en-us/products/liquid-chromatography/lc-supplies/capillaries-fittings/a-line-fittings/agilent-a-line

# InfinityLab Fittings & Special capillaries

The Quick Connect fitting is great for the column connection, where you connect and disconnect most often.

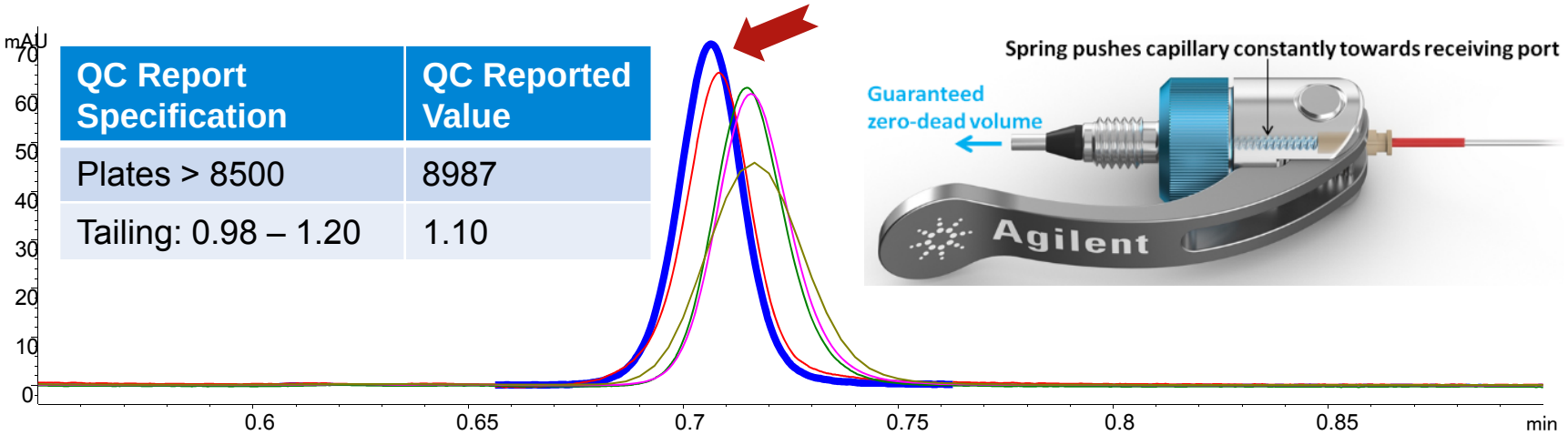

Quick Turn for other points, where there isn't room for the Quick Connect, e.g. the injector or detector


- A wide variety of capillary lengths and inner diameters are available to meet HPLC and UHPLC needs
- Capillaries and ferrules are completely user replaceable
- **Avoid** connection with InfinityLab fittings

# To Ensure the Best Performance of a 2.1 x 50 mm, 2.7 µm Poroshell 120 EC-C18 Column: Improve LC Capillary Connections

| System Modifications | Pressure (bar) | k' (naphthalene) | TF(naphthalene) | N(naphthalene) |
|---|---|---|---|---|
| Starting Method: DAD = 5 Hz | 159 | 2.3 | 1.06 | 3962 |
| Increase DAD to 20 Hz | 159 | 2.3 | 1.14 | 7786 |
| Increase DAD to 80 Hz | 159 | 2.3 | 1.15 | 8317 |
| Exchange flow path for 0.08 mm id cap + 0.6 µL flow cell | 188 | 2.4 | 1.00 | 9387 |
| **Install A-Line Fitting** | **182** | **2.4** | **1.00** | **9917** |

| QC Report Specification | QC Reported Value |
|---|---|
| Plates > 8500 | 8987 |
| Tailing: 0.98 – 1.20 | 1.10 |

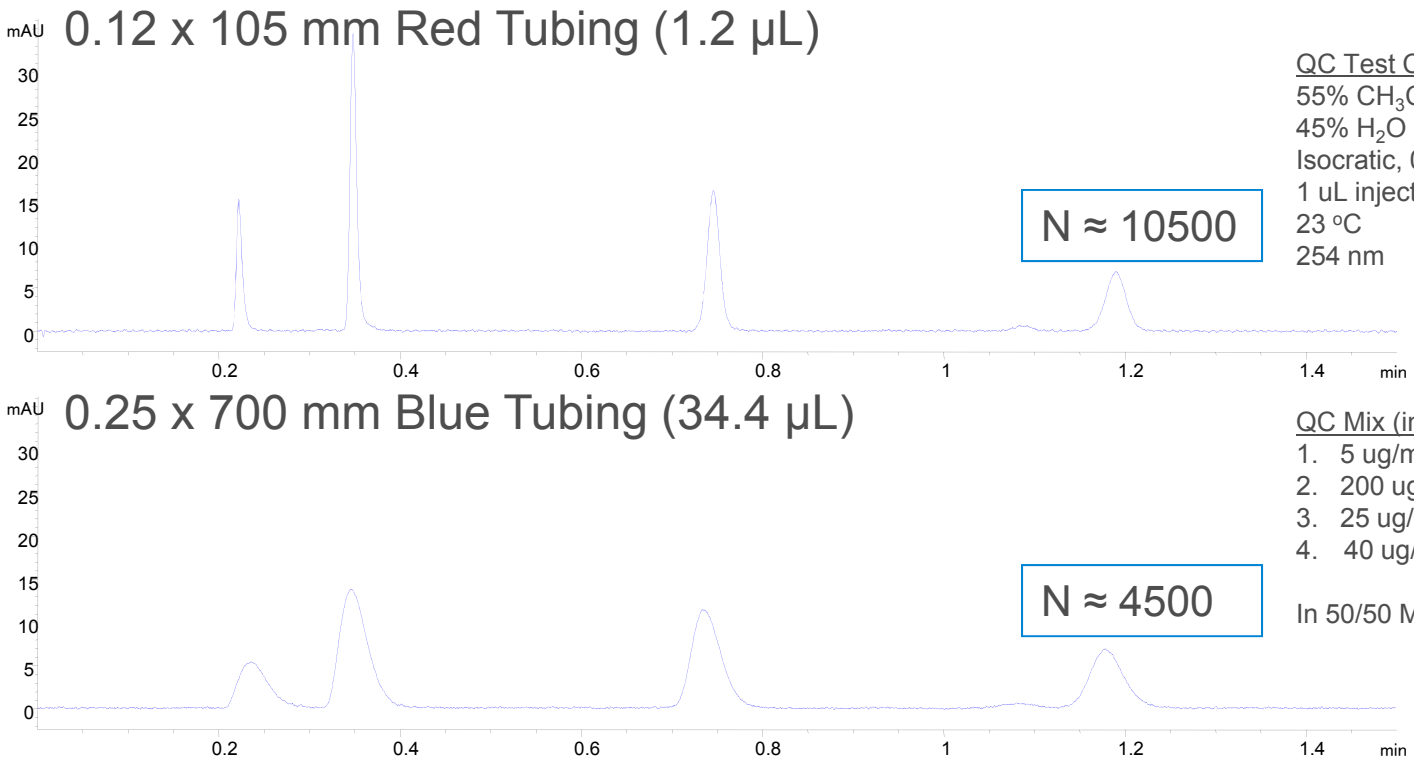






A: water, B: acetonitrile, 0.50 mL/min at 65% B, 0.5 µL injection of Newport QC standard), 80 µg/mL naphthalene, 25 °C, DAD: 254, 8 nm at 80, 20 or 5 Hz

# Do NOT Use Random Pieces of Tubing




QC Test Conditions:
55% $CH_3CN$
45% $H_2O$
Isocratic, 0.6 mL/min
1 uL injection of QC Mix
23 °C
254 nm

QC Mix (in elution order):
1. 5 ug/mL uracil
2. 200 ug/mL phenol
3. 25 ug/mL 4-chloro-nitrobenzene
4. 40 ug/mL naphthalene

In 50/50 MeCN/Water

➢ QC test of a 2.1 x 50 mm, 1.8-µm Eclipse Plus C18 showing peak broadening when larger volume tubing is installed between the autosampler and column. 43% of the efficiency lost with too much extra column volume

➢ **To avoid this pitfall** - Minimize extra column volume contribution from tubing by using the shortest length you can with the appropriate diameter and make proper connections

# Detector – Which One & Why

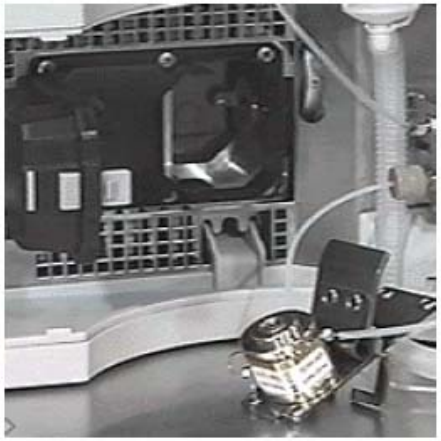
- UV/DAD
  - Popular, simple to use, reliable, sensitive
  - Sample must have UV absorbance
- MS
  - Sensitive
  - Sample must be ionizable
- RI
  - Refractive Index; difference between analyte and mobile phase
  - Need strict temperature control
- ELSD
  - Independent of a compound's absorbance, fluorescence, or electro-activity
  - Enables detection of semi-volatile and thermally sensitive compounds
- FLD
  - More selective and can be more sensitive
  - Compounds must fluoresce; Compounds often derivatized
- ECD
  - Very sensitive
  - Can produce severe noise

# UV Detector

- Sample
- Appropriate wavelength
  - Sample
  - Reference
    - Selecting Reference Wavelength in DAD Spectroscopy, http://www.chem.agilent.com/Library/Support/Documents/faq184.pdf
    - How To Select Reference Wavelength on DAD or MWD, http://www.chem.agilent.com/Library/Support/Documents/faq182.pdf
  - Mobile phase components
    - UV Cutoff
- Flow Cell
- Data rate
  - Column size
  - Response

# Flow Cells

## Match flow cell volume to chromatographic peak widths



| Flow Cell Volume/Pathlength | Uv Signal /Noise | Chrom. Resolution* |
|---|---|---|
| 13 µl / 10 mm | +++ | + |
| 5 µl / 6 mm | ++ | ++ |
| 1.7 µl / 6 mm | + | +++ |

* Depends on analytical conditions and column dimension

**13 µl Standard Flow Cell:**

For highest sensitivity and linearity

4.6-3 mm ID; 2.7, 3.5, 5 µm columns

**1.7 µl Micro Flow Cell:**

For highest resolution

UHPLC, 1.8, 2.7 µm

2.1-1 mm ID columns

**5 µl Semi-micro Flow Cell:**

Best compromise of sensitivity & selectivity

HPLC/UHPLC, 1.8 to 5 µm

4.6 – 1 mm ID columns

Other flow cells include:

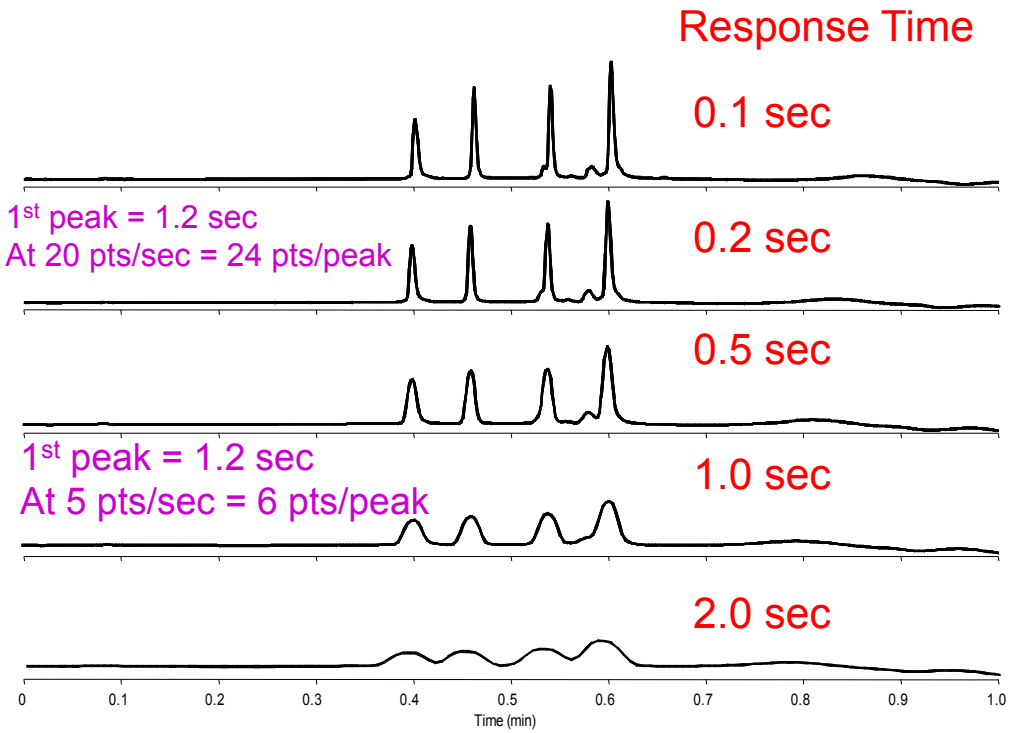
Max-Light Cartridge cells for Infinity DAD

500 nL for capillary LC

80 nL for nano LC

0.6 mm for Prep LC

# Effect of Detector Response Time on Fast Gradient Analyses




Agilent 1100 DAD
Agilent 1100 WPS with ADVR

Column: Poroshell 300SB-C18
2.1 x 75 mm, 5 mm

Mobile Phase:
A: 95% $H_2O$, 5% ACN with 0.1% TFA
B: 5% $H_2O$, 5% ACN with 0.1% TFA

Flow Rate: 2 mL/min

Temperature: 70°C

Detector: UV 215 nm

Piston stroke: 20

Sample:
1. Neurotensin 3. Lysozyme
2. RNaseA 4. Myoglobin

- You may have to adjust the response rate of your detector for rapid peak detection
- To avoid missing the peak, make sure detector is set properly
  - Need ~25 data points to accurately "describe" a chromatographic peak.

http://www.chem.agilent.com//Library/Support/Documents/FAQ_Approved_PDF_Template_enough_datapoints.pdf

# Column 

## Cautions and Potential Pitfalls

- Performance Report
- Not all C18 (L1) columns are the same
- What kills a column
- Pressure
- Lot-to-lot

# Performance Report

**SERIAL NUMBER: USDAZ01333**

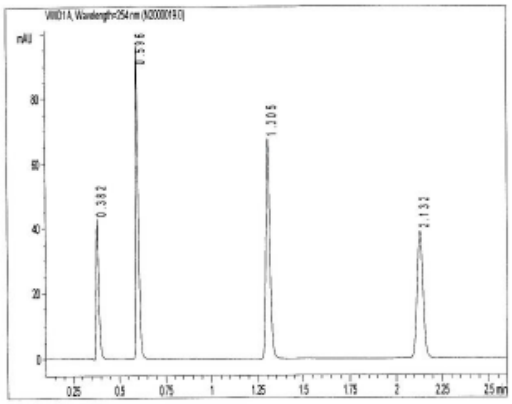
PART NUMBER: 959758-902
COLUMN TYPE: ZORBAX RRHD Eclipse Plus C18 2.1 x 100 mm, 1.8 µm
PACKING LOT #: B09089

**TEST CONDITIONS**

| | | |
|---|---|---|
| MOBILE PHASE | = | 60% Acetonitrile / 40% Water |
| COLUMN PRESSURE | = | 517.2 Bar |
| COLUMN FLOW | = | 0.50 ml / min |
| LINEAR VELOCITY | = | 0.436 cm / sec |
| TEMPERATURE | = | AMBIENT (Nominally 23 °C) |
| INJECTION VOLUME | = | 1 µl |

**QUALITY CONTROL PERFORMANCE RESULTS FOR NAPHTHALENE**

| | TEST VALUES | SPECIFICATIONS |
|---|---|---|
| THEORETICAL PLATES = | 22337 | MIN = 21000 |
| SELECTIVITY = | 1.90 | RANGE = 1.82 - 1.92 |
| USP TAILING FACTOR = (@ 5% Peak Height) | 1.08 | RANGE = 0.98 - 1.20 |
| k' = | 4.58 | |




***Sample components with concentrations diluted in mobile phase in the following elution order.***

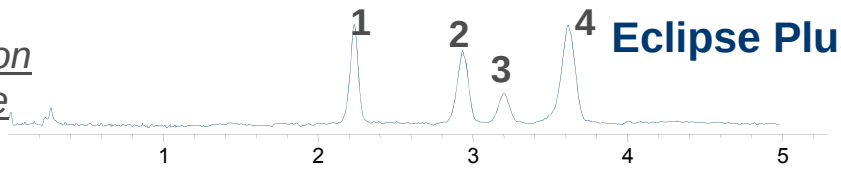
| Peak # | Conc (ug/ml) | Sample Component |
|---|---|---|
| 1 | 10 | Uracil |
| 2 | 400 | Phenol |
| 3 | 50 | 4-Chloro Nitrobenzene |
| 4 | 80 | Naphthalene |

- Manufacturing test chromatogram is done on a modified LC system to minimize extra column volume and will differ from a typical lab instrument

- Don't expect to get the exact same result as the performance report
- Test column performance on your instrument to have as a reference

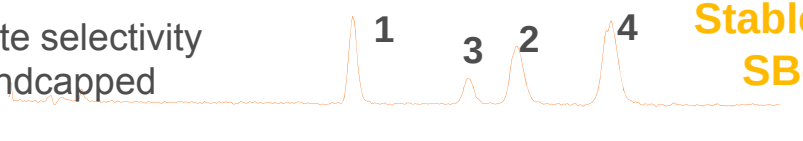
# Not All C18s Are The Same

**1st choice**
*Best Resolution & Peak Shape*



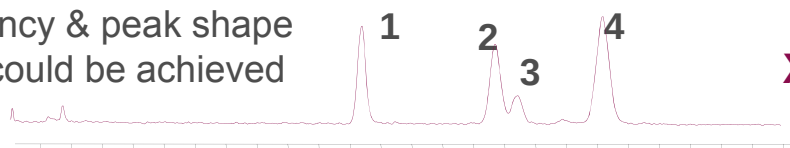

**Eclipse Plus C18**

**2nd choice**
Good alternate selectivity due to non-endcapped



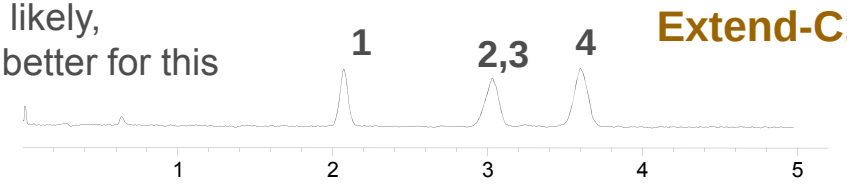

**StableBond SB-C18**

**3rd choice**
Good efficiency & peak shape
Resolution could be achieved




**Eclipse XDB-C18**

**4th choice**
Resolution not likely,
Other choices better for this separation.




**Extend-C18**

- Multiple bonded phases for most effective method development
- Match to one you are currently using
- *Method development kits are available*

Mobile phase: (69:31) ACN: water
Flow 1.5 mL/min.
Temp: 30 °C
Detector: Single Quad ESI positive mode scan
**Columns: RRHT 4.6 x 50 mm 1.8 um**

Sample:
1. anandamide (AEA)
2. Palmitoylethanolamide (PEA)
3. 2-arachinoylglycerol (2-AG)
4. Oleoylethanolamide (OEA)

➢ **Avoid this pitfall** - Don't assume every C18 will behave the same

# Column Lifetime

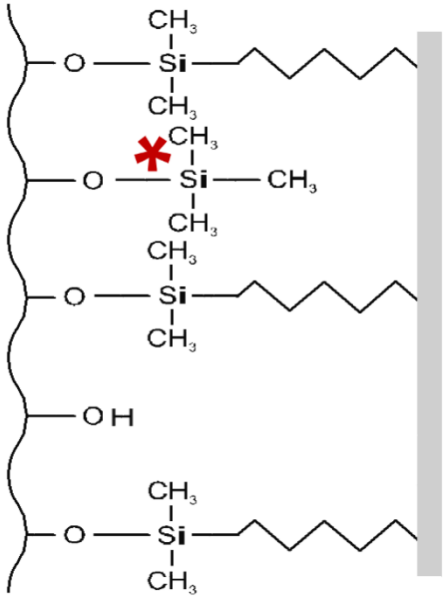
- Follow manufacturer's guidelines
- Method conditions that can affect column lifetime
  - pH
    - Low pH
      - pH<3 – acid hydrolysis of bonded phase
      - Retention time changes, resolution changes, increased peak tailing
    - High pH
      - Silica-based packing has some solubility at higher pH
  - Temperature
    - Poor temp choice can accelerate the dissolution of the silica above pH 6.
  - Buffer Choice
    - Avoid phosphate & carbonate buffers at high pH
  - Crud
    - Column contamination
    - Plugged frit
- Storage conditions
  - Columns should not be maintained at elevated pH or elevated temperature when not in use

# Operational Conditions

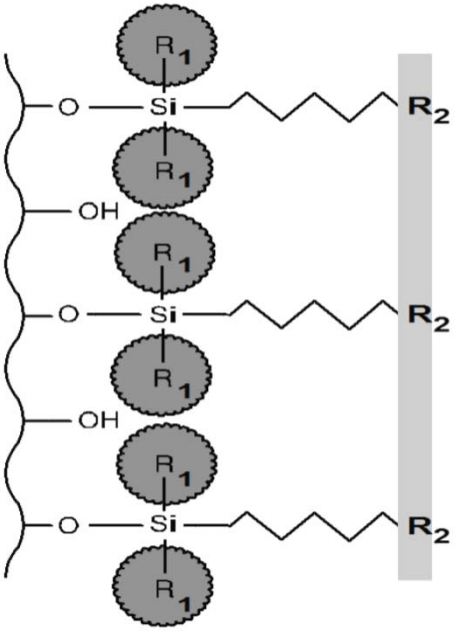
## Mobile Phase Effects on Column Life

### Low pH (1-3) - Bonded Phase Loss by Acid Hydrolysis

**Conventional**



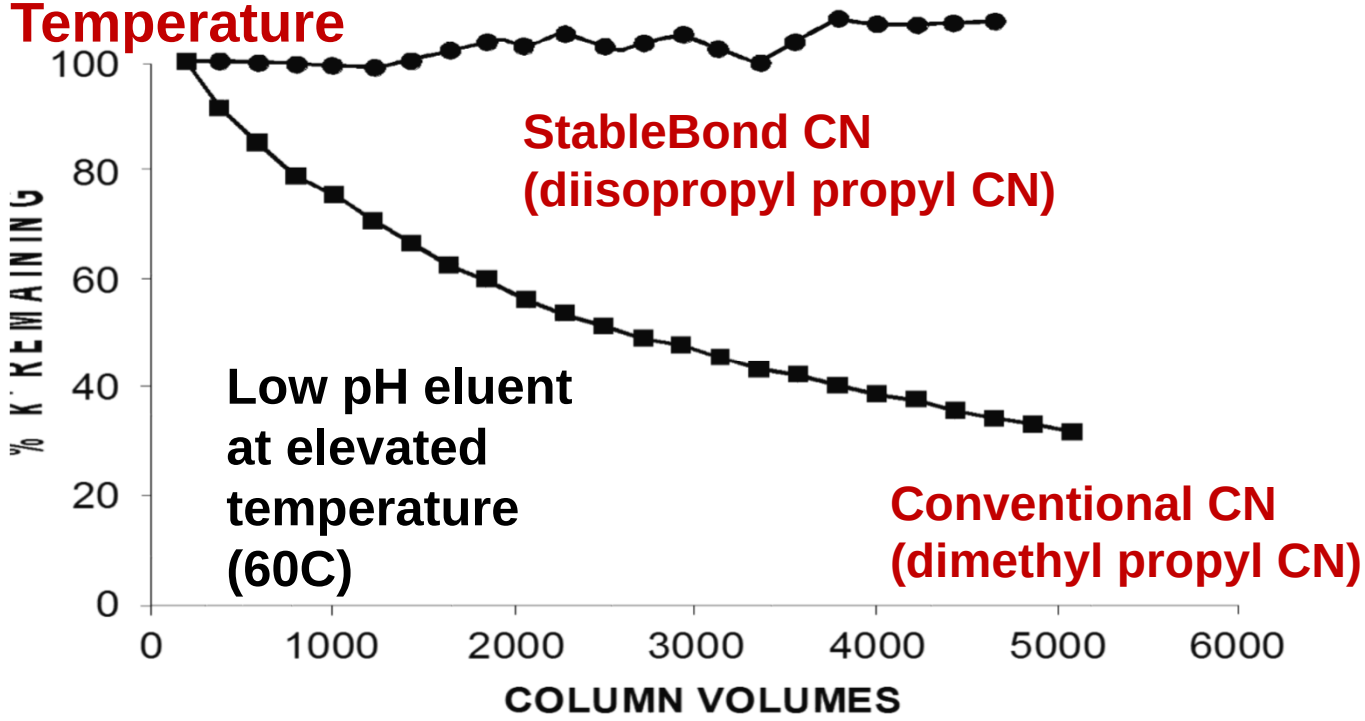

**StableBond**



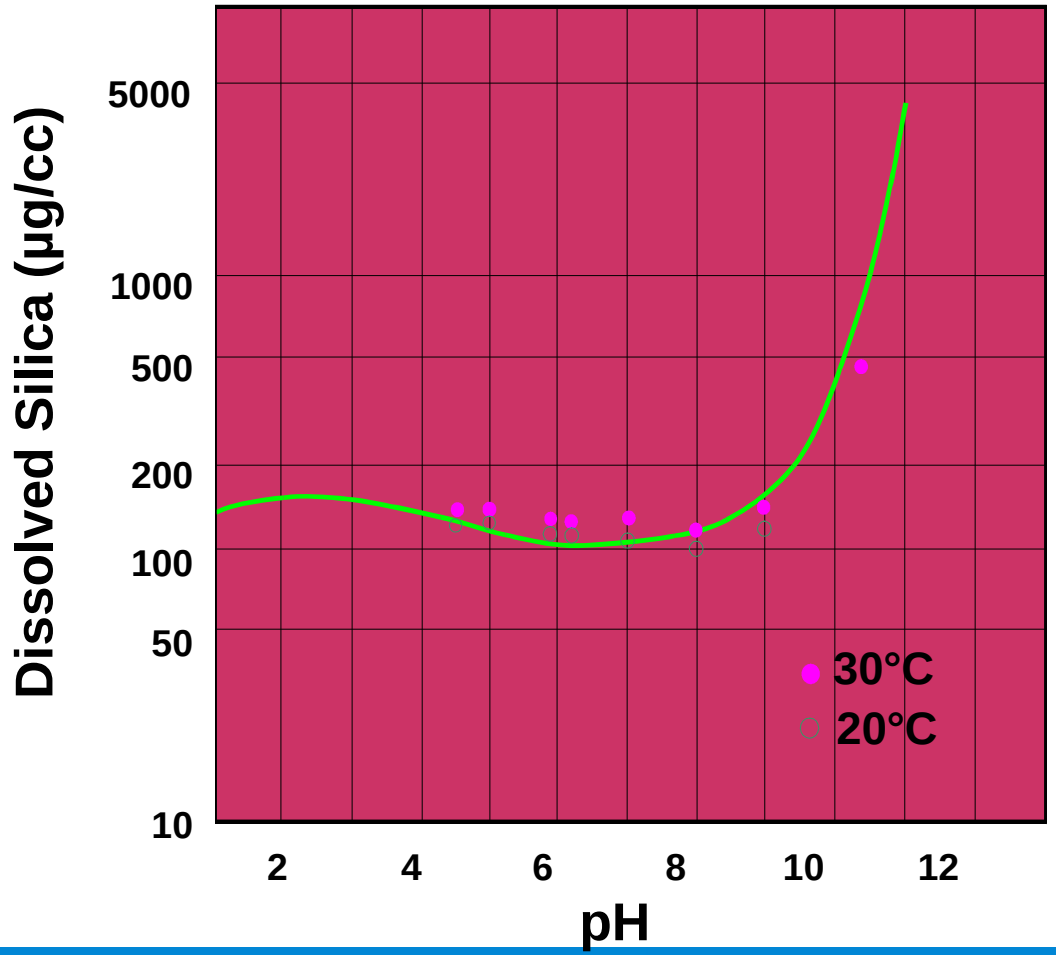
# Operational Conditions

## Mobile Phase Effects on Column Life

**Bonded-Phase Hydrolysis Rate Increases With Temperature**



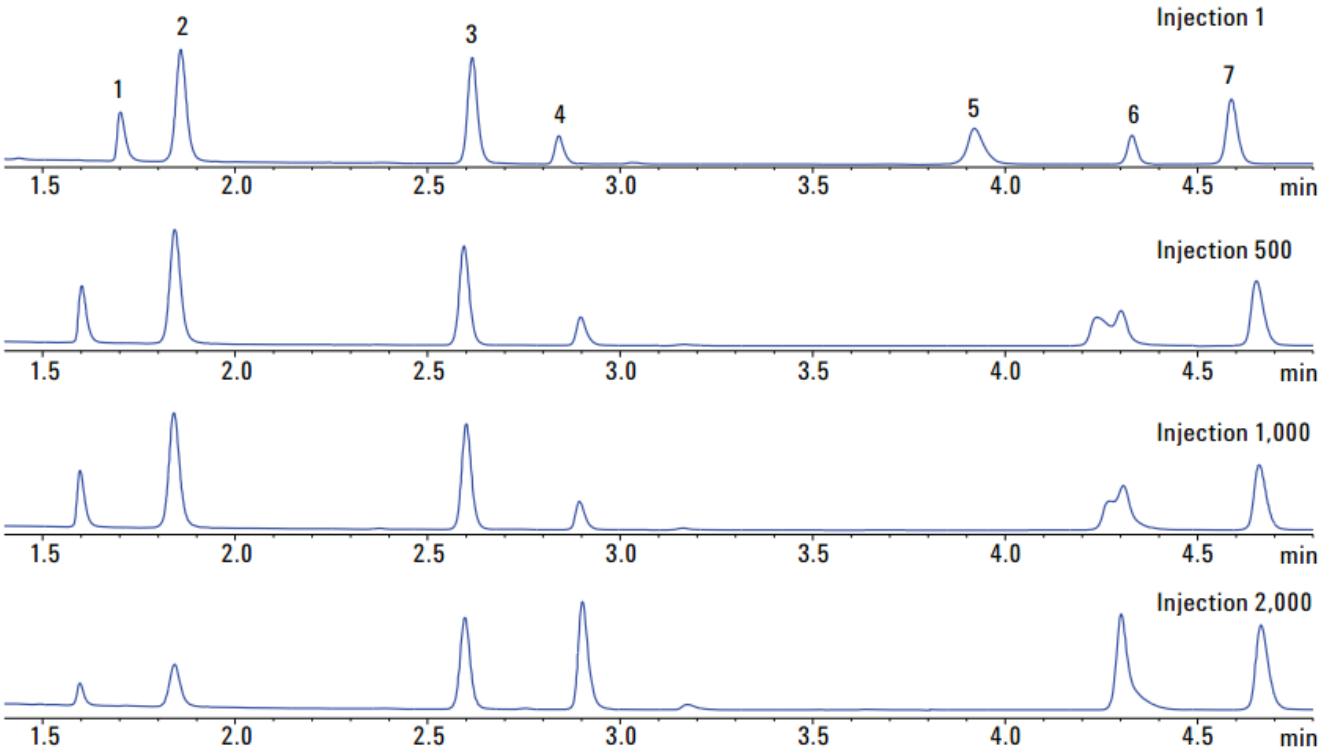
# Solubility of Amorphous Silica at Different pH Values




**Solubility of silica increases dramatically above pH 8.0.**

**Column bonding chemistry plays a crucial role in stability of silica at high pH**

# Column Lifetime with High pH Methods




Other brand column on an ammonium bicarbonate gradient at pH 10.

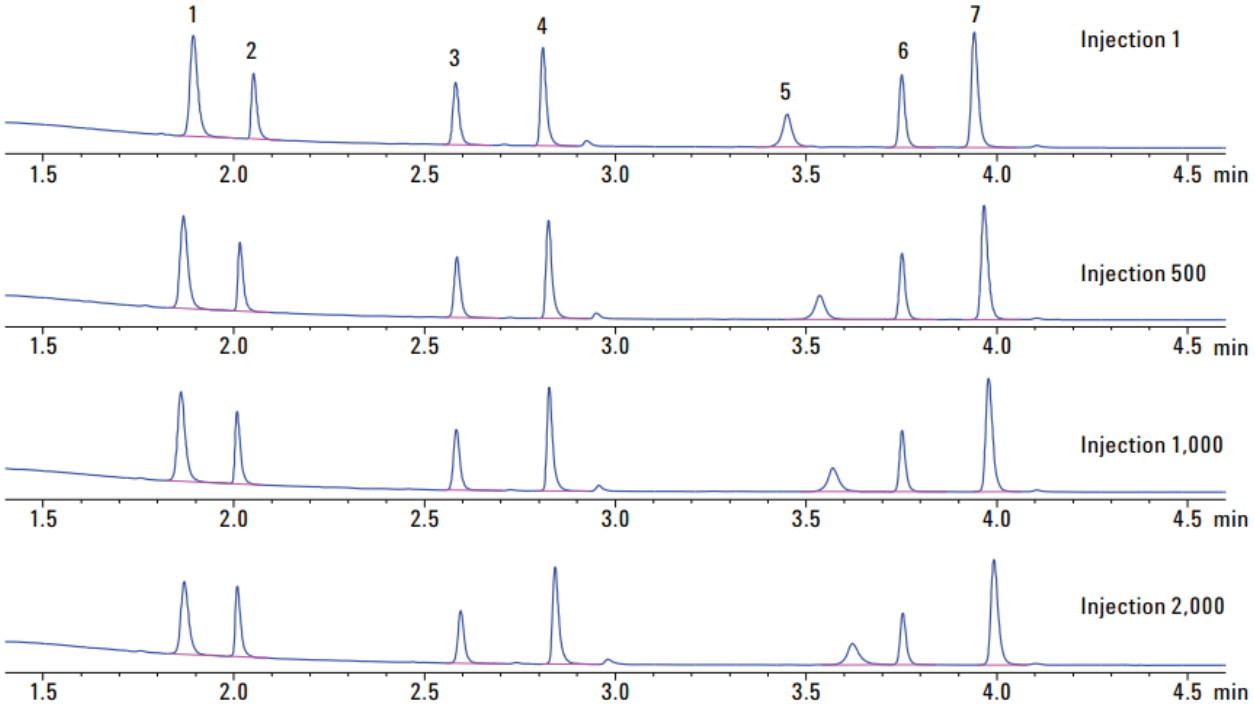
**Peak ID**

1. Methyl salicylate
2. 4-Chlorocinnamic acid
3. Acetophenone
4. Quinine
5. Nortriptyline
6. Heptanophenone
7. Amitriptyline

**Conditions**

| | | |
|---|---|---|
| Column: | Other brand, 2.1 x 50 mm, 3 µm | |
| Eluent: | A) 10 mM ammonium bicarbonate adjusted to pH 10.0 in water<br>B) acetonitrile | |
| Flow rate: | 0.4 mL/min | |
| Gradient: | Time (min) | %B |
| | 0 | 5 |
| | 5 | 95 |
| | 5.1 | 5 |
| Total run time: | 7 min | |

# Column Lifetime with HPH-C18




**Peak ID**

1. Methyl salicylate
2. 4-Chlorocinnamic acid
3. Acetophenone
4. Quinine
5. Nortriptyline ← pKa 9.7
6. Heptanophenone
7. Amitriptyline

**Conditions**

| | |
|---|---|
| Column: | Agilent Poroshell 120 HPH-C18, 2.1 × 50 mm, 2.7 µm |
| Eluent: | A) 10 mM ammonium bicarbonate adjusted to pH 10.0 in water<br>B) acetonitrile |
| Flow rate: | 0.4 mL/min |
| Gradient: | Time (min) %B<br>0 5<br>5 95<br>5.1 5 |
| Total run time: | 7 min |

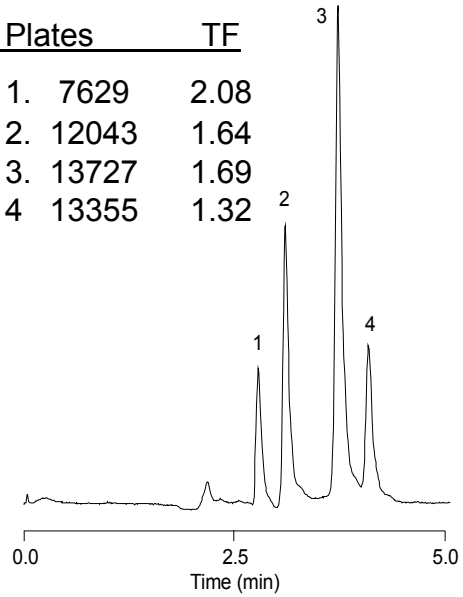
← *Same Conditions*

- **Avoid heartbreak** – Choose the right column for your conditions

# Peak Tailing from Column Contamination (CRUD)

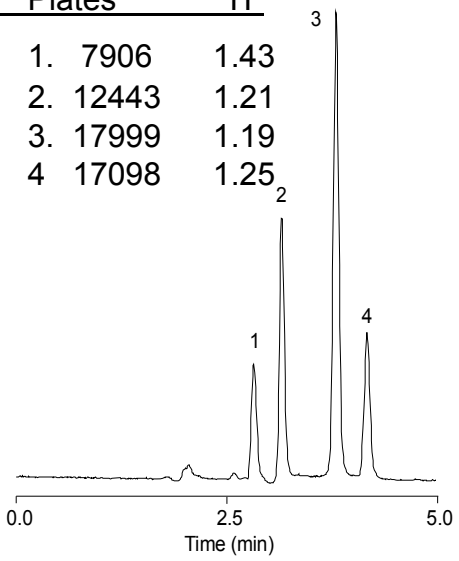
Column: StableBond SB-C8, 4.6 x 250 mm, 5µm  Mobile Phase: 20% $H_2O$ : 80% MeOH  Flow Rate: 1.0 mL/min
Temperature: R.T.  Detection: UV 254 nm  Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

| | Plates | TF |
|---|---|---|
| 1. | 7629 | 2.08 |
| 2. | 12043 | 1.64 |
| 3. | 13727 | 1.69 |
| 4 | 13355 | 1.32 |



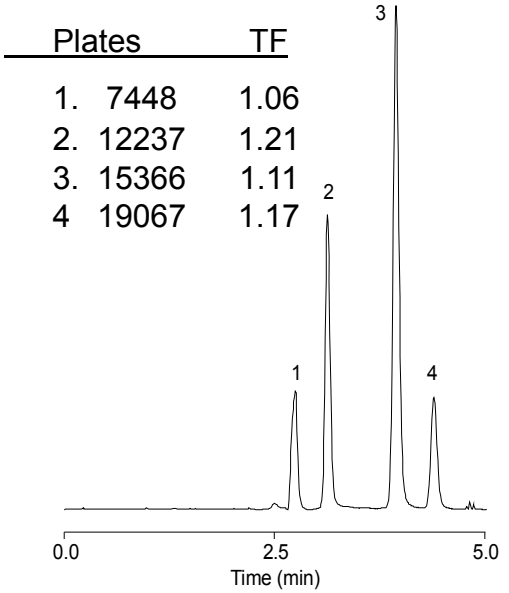

QC test forward direction

| | Plates | TF |
|---|---|---|
| 1. | 7906 | 1.43 |
| 2. | 12443 | 1.21 |
| 3. | 17999 | 1.19 |
| 4 | 17098 | 1.25 |




QC test reverse direction

| | Plates | TF |
|---|---|---|
| 1. | 7448 | 1.06 |
| 2. | 12237 | 1.21 |
| 3. | 15366 | 1.11 |
| 4 | 19067 | 1.17 |




QC test after cleaning
100% IPA, 35°C

- Good column hygiene can extend the life of your column
  - Column cleaning procedure (see appendix)

# Preventing Column Pressure Problems

1. Filter mobile phase:
   - filter non-HPLC grade solvents
   - filter buffer solutions
   - Install an in-line filter between auto-sampler and column (removes pump seal debris, injector rotor debris, and sample particulates). Use 2 um frit for 3.5 um/5um columns, use 0.5 um (or smaller) frit for 1.8 um/2.7 columns
2. Filter all samples and standards

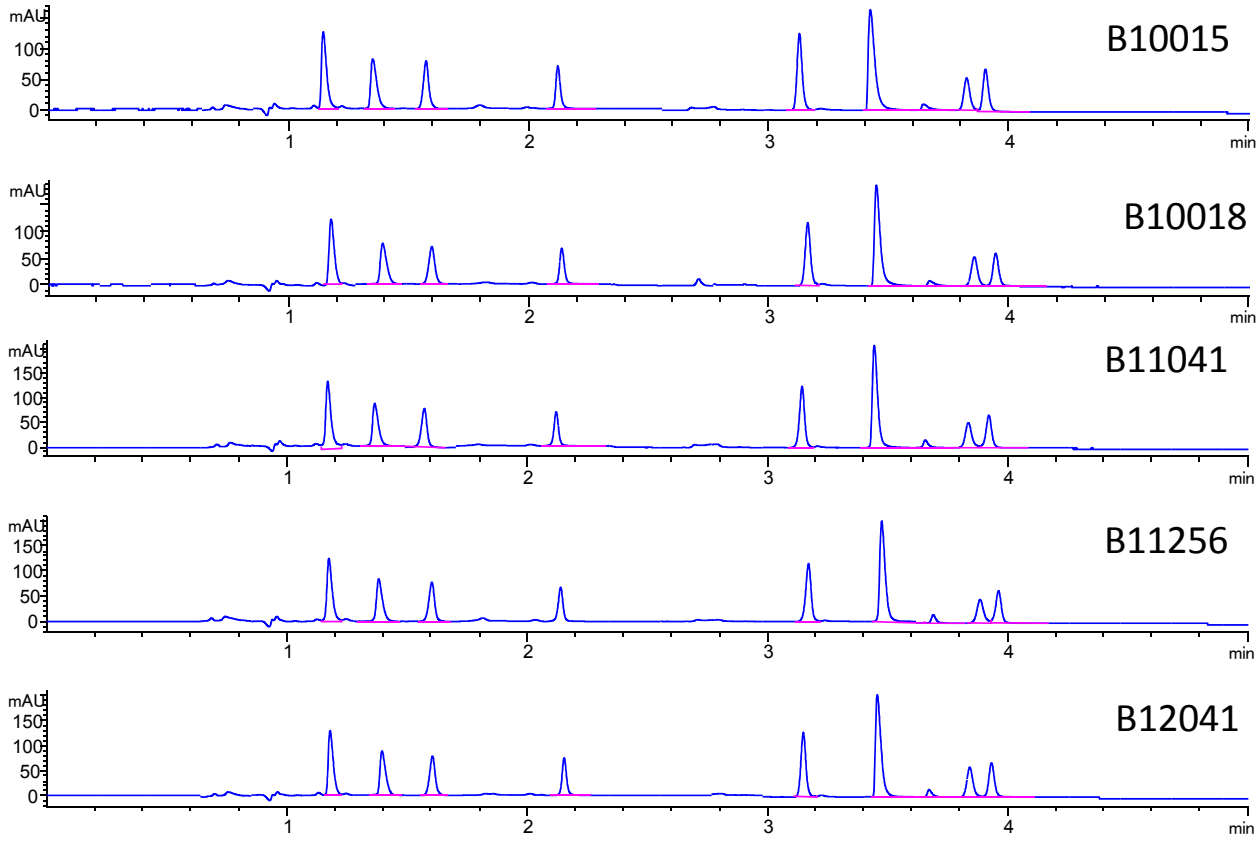
3. Perform sample clean-up (i.e. SPE, LLE) on dirty samples.
4. Appropriate column flushing – flush buffers from entire system with water/organic mobile phase
5. Replace buffers every 24-48 hours, never add to the bottle, always use a new one

➢ **Avoid** pressure pitfalls – Filter Samples

# Lot-to-Lot Comparison of 5 Lots
# Poroshell 120 EC-C18

2010

2012

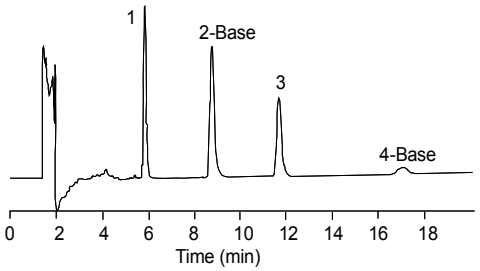



A 20 mM Ammonium Acetate pH 4.80 adjusted with 20 mM Acetic Acid B: Acetonitrile, 30 C, Sig= 230 nm,4: ref 360,100
0.638 mL / min 10% to 40% B over 4 min, 3 x100 mm Poroshell 120 EC-C18
2ul Ascorbic acid, acesulfane K, saccharin, caffeine, aspartame ,sorbic acid , quinine, dehydroacetic acid

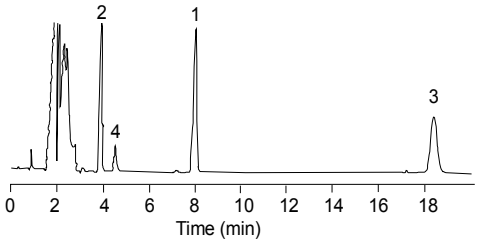
Agilent InfinityLab Poroshell 120 Columns for HPLC and UHPLC (Pub# 5991-7435EN)
Agilent InfinityLab Poroshell 120 Columns 1.9um Columns for UHPLC (Pub# 5991-7352EN)

# Lot-to-Lot Selectivity Change Related to pH Choice

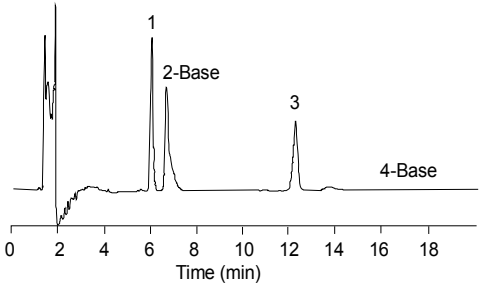
**pH 4.5 - Lot 1**



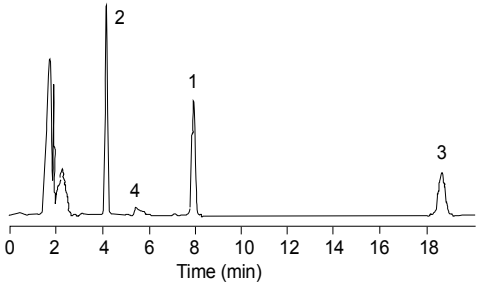

**pH 3.0 - Lot 1**




**pH 4.5 - Lot 2**




**pH 3.0 - Lot 2**




- pH 4.5 - selectivity change from lot-to-lot for basic compounds
- pH 3.0 - no selectivity change from lot-to-lot

- Avoid  - *Do not validate on only 1 column lot!!!!!!!!!*

- For Method Ruggedness
  - oTest 3 different column lots
  - oCompare $R_s$ for the 3 lots
    - •If $\Delta R_s$ is too large, modify method

# Method Conditions
## Cautions and Potential Pitfalls

- Mobile phase
  - What's in your mobile phase and why
  - Don't believe everything you read; Focus of paper may not be chromatography
  - Microbial growth
  - Baseline
    - Drift
      - Additives, e.g., TFA
      - Detector, e.g., RI
    - Problems, http://www.chem.agilent.com/Library/Support/Documents/Baseline_problems.pdf
  - Mobile phase preparation
  - Ghost Peaks
    - Sample
    - Mobile phase components; $H_2O$, solvent, additives
- pH
- Temperature
- Injection problems

# Microbial Growth

## ➢Potential problems

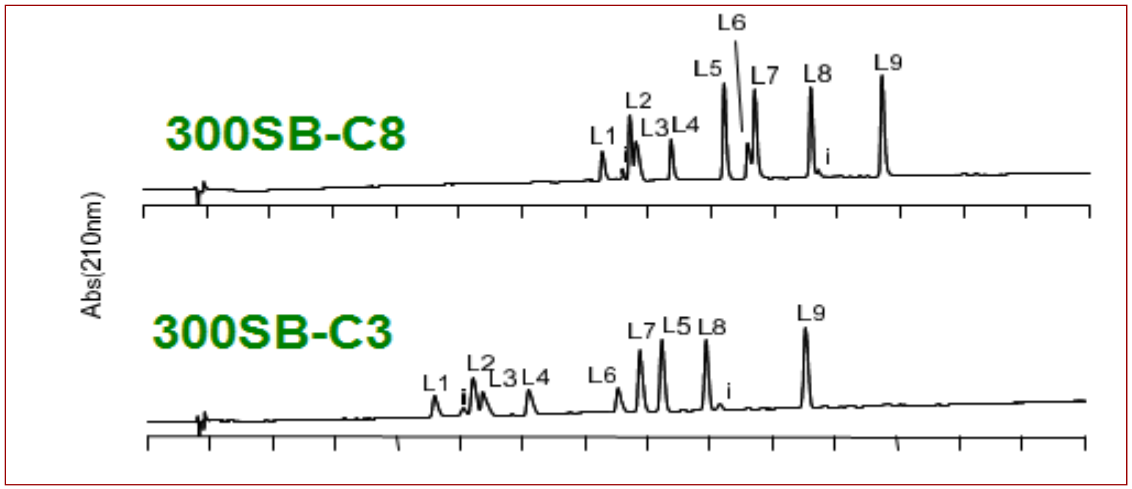
- Increased system pressure or pressure fluctuations
- Increased column pressure, premature column failure
- Can mimic application problems
- Gradient inaccuracies
- Ghost peaks
- Difficult to remove if gets in degasser and rest of system

## ➢Prevent and/or Reduce Microbial Growth

- Use freshly prepared mobile phase
- Filter
- Do not leave mobile phase in instrument for days without flow
- Always discard “old” mobile phase
  - o Do not add fresh mobile phase to old
- Use an amber solvent bottle for aqueous mobile phase
- If possible, can add
  - o 5% organic added to water can be used to reduce bacterial growth
  - o Few mg/l sodium azide

➢To avoid contaminating your system and column, prevent microbial growth
- Check your instrument manual for guidelines

# Baseline Drift




## Conditions:

Columns: ZORBAX 300SB, 4.6 x 150 mm, 5 µm
Mobile Phase: Gradient, 0 - 26% B in 30min.
A = 0.1% TFA in Water
B = 0.1% TFA in Acetonitrile
Temperature: 40°C
Sample: 2 µg of each peptide
Flow Rate: 1.0 mL / min.
Detection: UV-210nm

➢ Know the UV Cutoff of your mobile phase components

# Mobile Phase Preparation

- HPLC grade or better
- Buffer preparation procedure
  - Be consistent
  - Document the process
- Volume % of solvents can depend on preparation

Specified volume ACN added to a 1 L volumetric and made to volume with $H_2O$

≠

Specified volume $H_2O$ added to a 1 L volumetric and made to volume with ACN

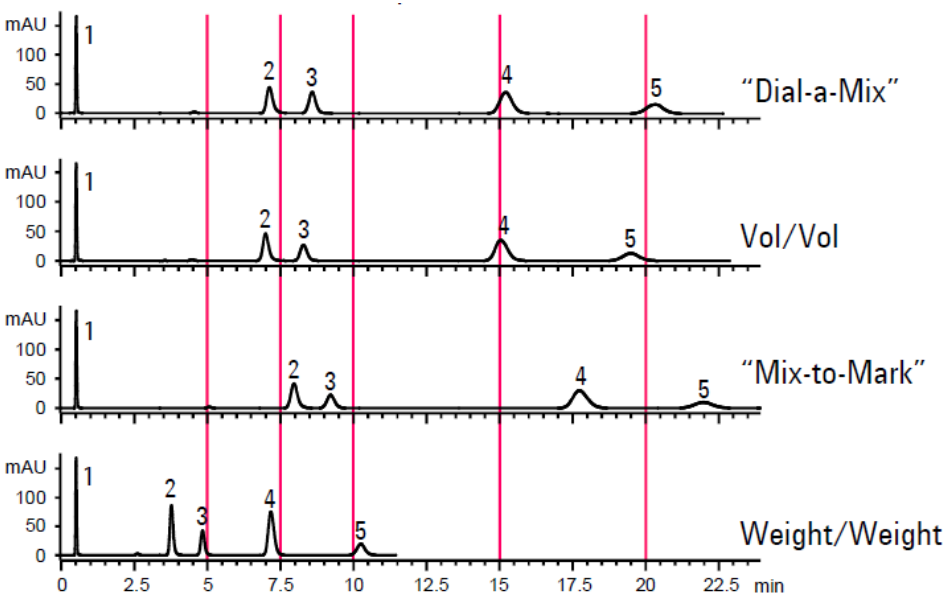
≠

500 ml $H_2O$ added to 500 ml ACN

- Small changes in mobile phase strength can have a large effect on retention

# Mobile Phase Preparation

## Effect on Chromatography




| | |
|---|---|
| HPLC System: | Agilent 1100 with quaternary pump |
| Column: | ZORBAX Eclipse XDB-C8 Rapid-Resolution (3.5µm), 4.6 x 50 mm<br>Agilent Part No. 935967-906 |
| Mobile Phases: | Dial-a-Mix= A: water B: MeOH, pump 50% B<br>Vol/Vol=250 mL water + 250 mL MeOH, pump 100%<br>Mix-to-Mark = 250 mL MeOH, fill to 500 mL with water, pump 100%<br>Premixed (w/w) = 200 g MeOH + 200 g water, pump 100% |
| Detection: | UV 254 nm |
| Flow: | 1 mL/ min. |
| Temperature: | ambient |

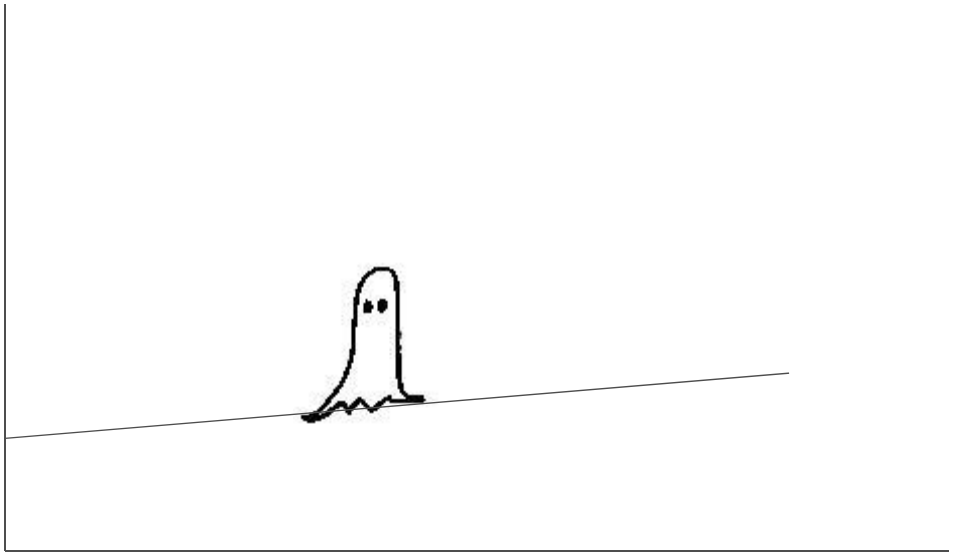
1. Uracil
2. Butylparaben
3. Napthalene
4. Dipropylphthalate
5. Acenaphthene

- Method used to prepare MP can significantly affect the elution pattern
- To avoid this potential pitfall
  - Be consistent
  - w/w is more accurate than v/v

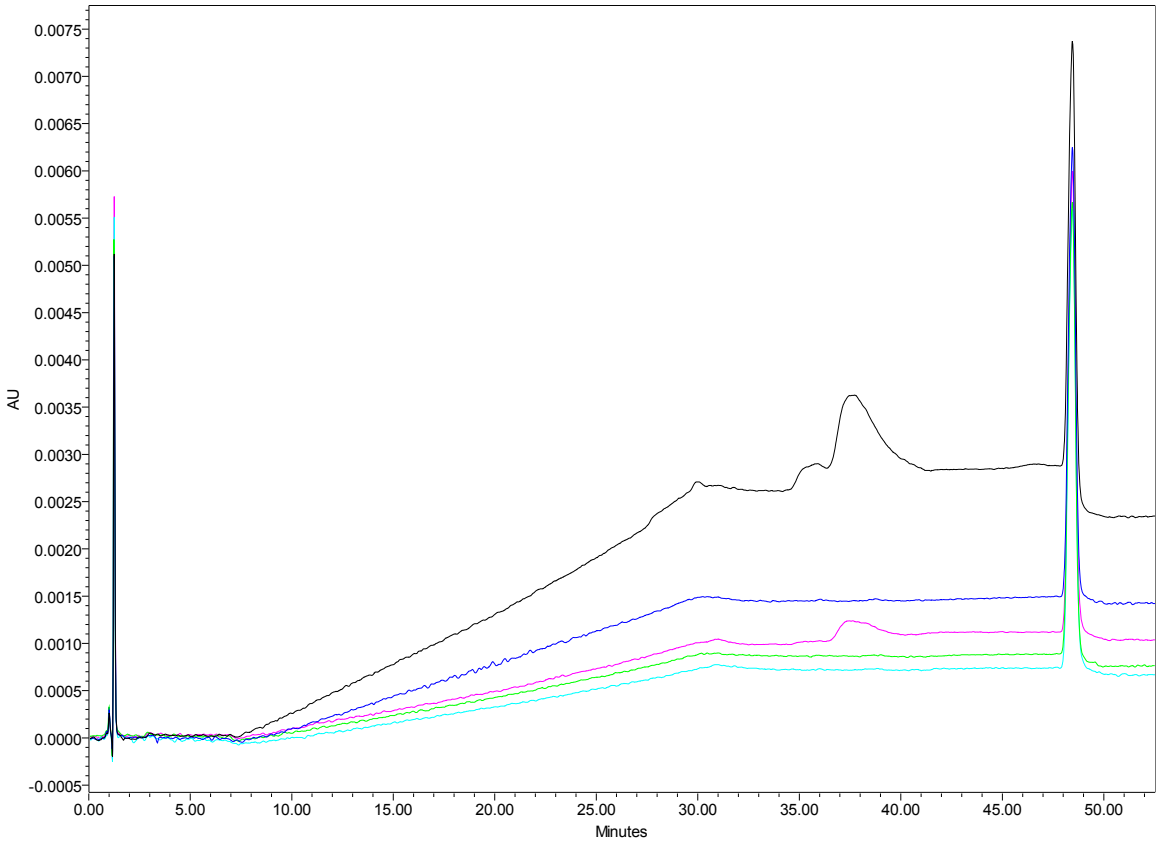
Effect of Mobile Phase Preparation on Chromatography,
Pub. No. 5988-6476EN

# Ghost Peaks
## Where Do They Come From

- Organic
- Additives
  - TFA
  - Salts
- $H_2O$
- Sample
- Other



# Acetonitrile Comparison

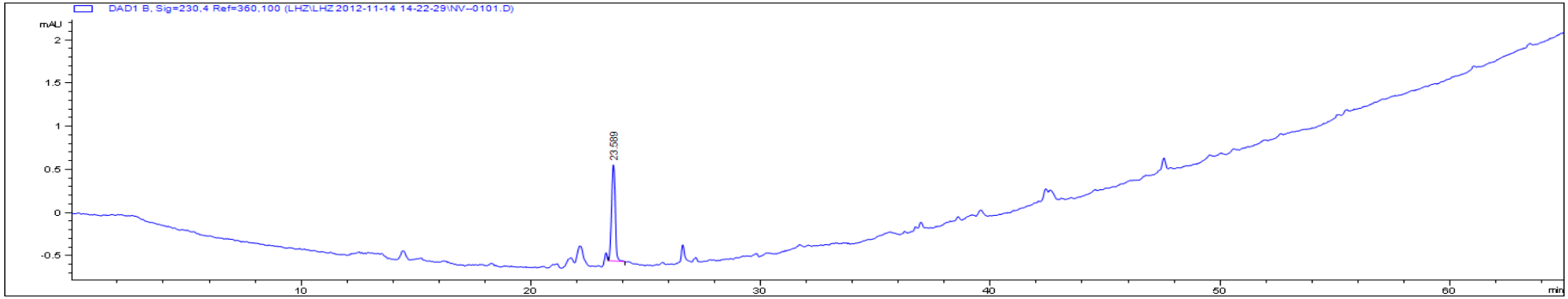



Multiple suppliers and lots of ACN tested

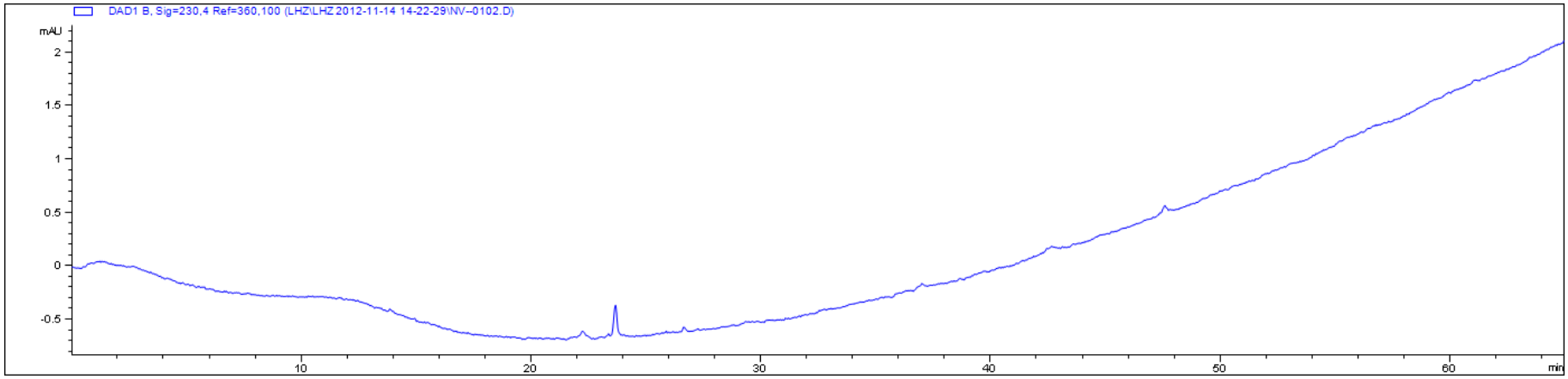
- Solvent - quality and consistency

# Ghost Peaks

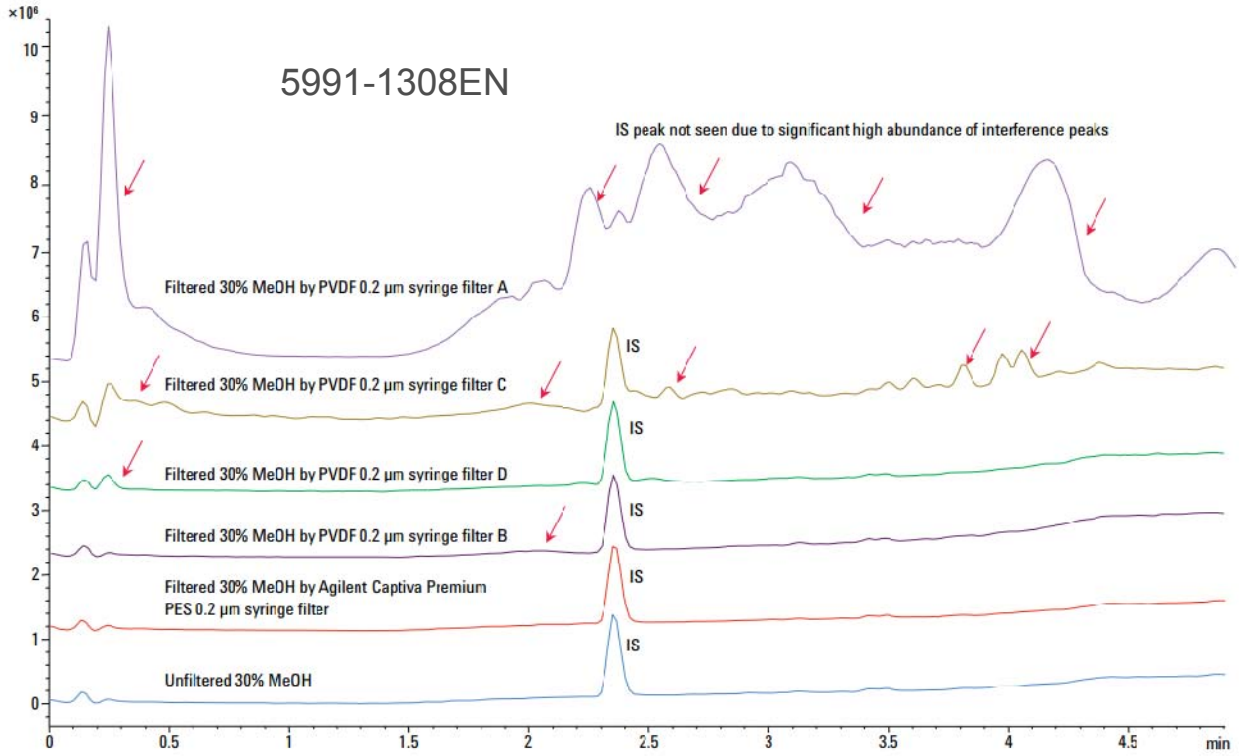
The LC system was equilibrated at starting conditions for 30min, then a gradient run was made. Impurities were trapped and eluted out with the gradient.




When an injection is made with minimal equilibration, a much cleaner baseline was observed.



# Choosing the Best Syringe Filters




Filter cleanliness comparison of the Agilent Captiva Premium PES syringe filter with non-Agilent PVDF syringe filters using LC/MS under positive mode.

| | |
|---|---|
| UHPLC: | Agilent 1290 Infinity LC System |
| Column: | Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (p/n 959757-902) |
| Mobile phases: | A: $H_2O$ + 0.01% formic acid (FA)<br>B: Acetonitrile + 0.01% FA |
| Flow rate: | 0.5 mL/min, gradient |
| Total run time: | 5 min plus 1 min post run |
| Gradient: | Hold at 30% B for 1 min, then ramped to 90% B in 3 min, and hold at 90% B for 1 min |
| Injection volume: | 8 µL |
| Internal standard: | 50 µg/mL Naproxin |
| MS: | Agilent 6150 Single Quadrupole LC/MS System |
| Source: | ESI with Agilent Jet Stream Technology (AJS-ES) |
| Capillary voltage: | 4,000 V |
| Nozzle voltage: | 2,000 V |
| Drying gas flow: | 12 L/min |
| Drying gas temp: | 250 °C |
| Nebulizer pressure: | 35 psig |
| Sheath gas flow: | 3.0 L/min |
| Sheath gas temp: | 150 °C |
| Mass range: | 100 – 1350 *m/z* |
| Fragmentor: | 150 V (pos), 80 V (neg) |

- Select a syringe filter appropriate for your sample and solvent used
- **Make sure** it does not add unwanted interferences

http://filtrationselectiontool.chem.agilent.com

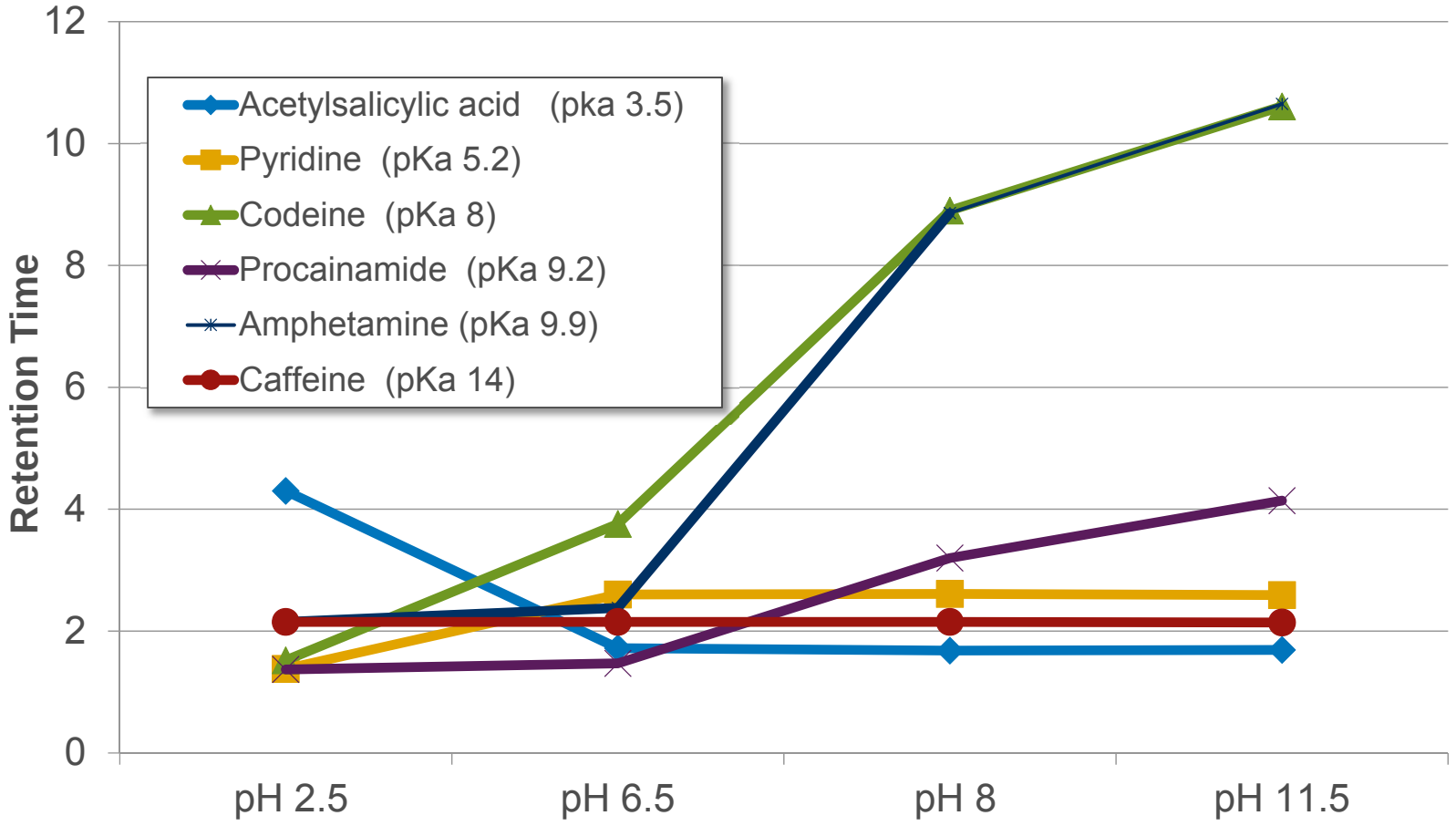
Captiva Filtration Selection Guide: 5991-1230EN

# Method Conditions

- Mobile phase
  - What's in your mobile phase and why
  - Don't believe everything you read; Focus of paper may not be chromatography
  - Microbial growth
  - Baseline
    - Drift
      - Additives, e.g., TFA
      - Detector, e.g., RI
    - Problems, http://www.chem.agilent.com/Library/Support/Documents/Baseline_problems.pdf
  - MP prep
  - Ghost Peaks
    - Sample
    - Mobile phase components; $H_2O$, solvent, additives
- pH
  - High
  - Low
- Temperature
- Injection problems
  - Sample solvent
  - Injection volume

# Change in Retention with pH for Ionizable Compounds is Compound-Dependent

**More retention for non-charged analytes (i.e. acids at low pH and bases at high pH)**



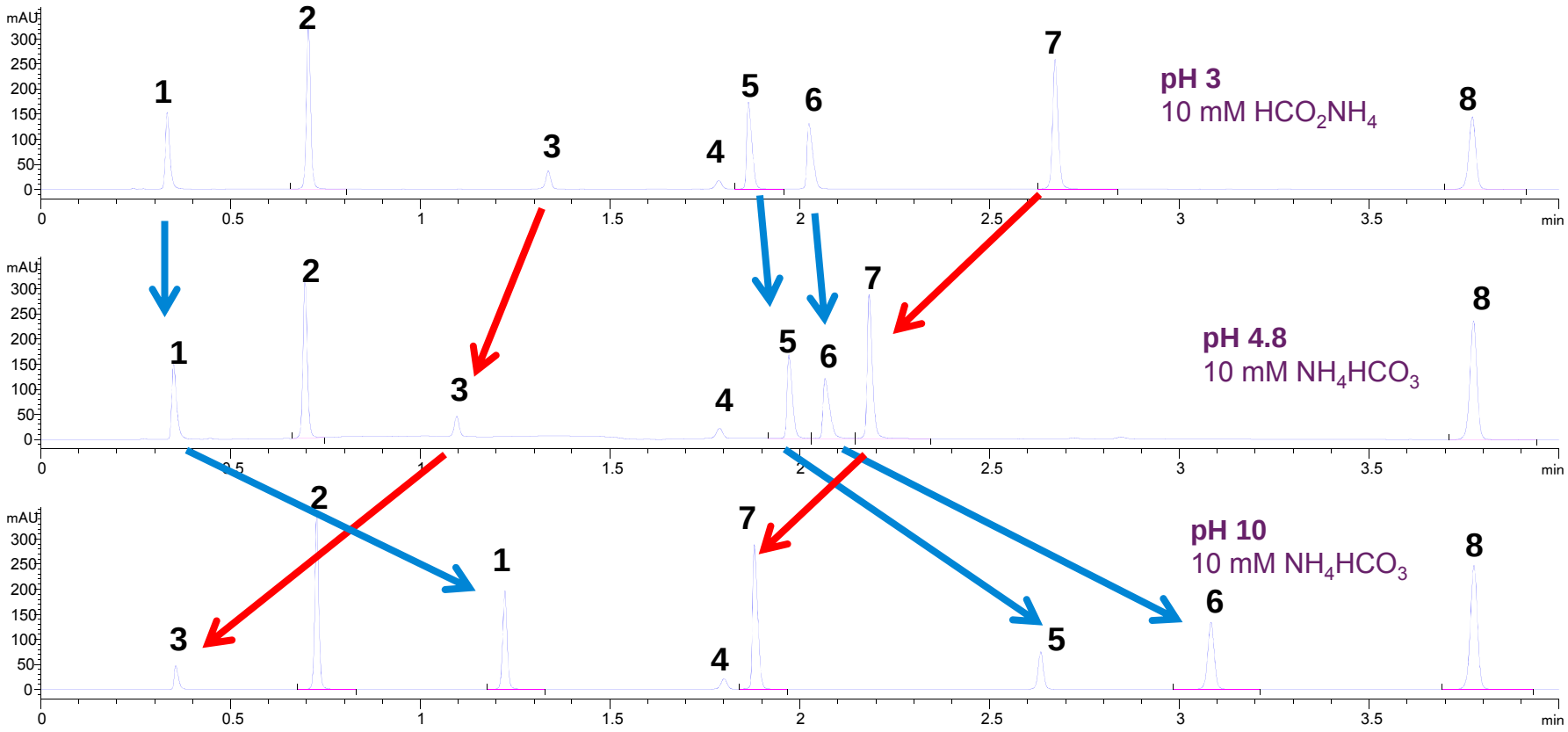

Mobile Phase: 45% MeOH, 55% 20 mM Phosphate Buffer

# pH can Affect Your Separation

| Time | % Buffer | % MeCN |
|---|---|---|
| 0 | 10 | 90 |
| 5 | 90 | 10 |
| 7 | 10 | 90 |
| 2 ml/min | | 254 mn |

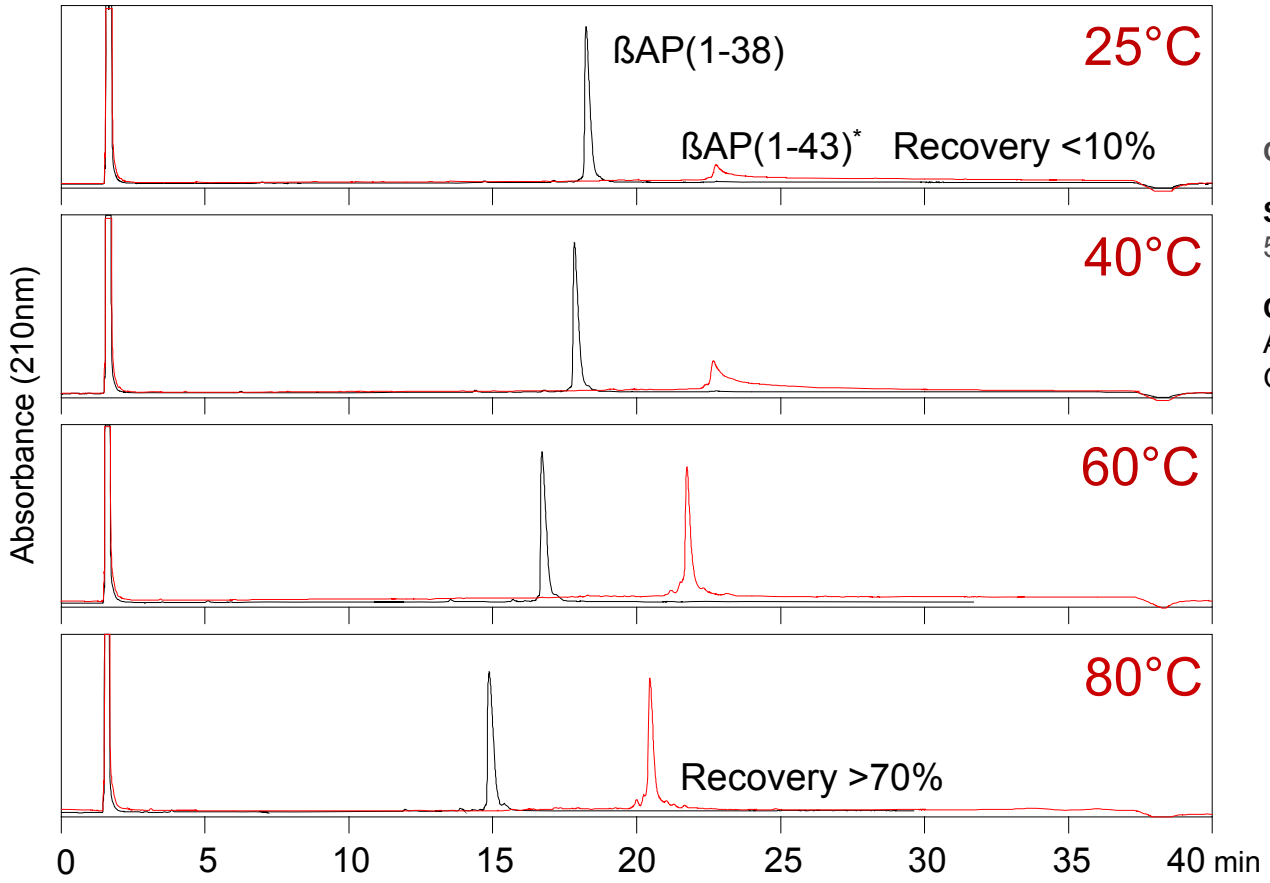
1. Procainamide
2. Caffeine
3. Acetyl Salicylic Acid
4. Hexanophenone Deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone

## Poroshell HPH-C18 4.6 x 50 mm, 2.7 µm




- **Know** if pH affects the retention of your analytes

# Temperature




**Column**: ZORBAX 300 SB-C18, 4.6 x 150 mm

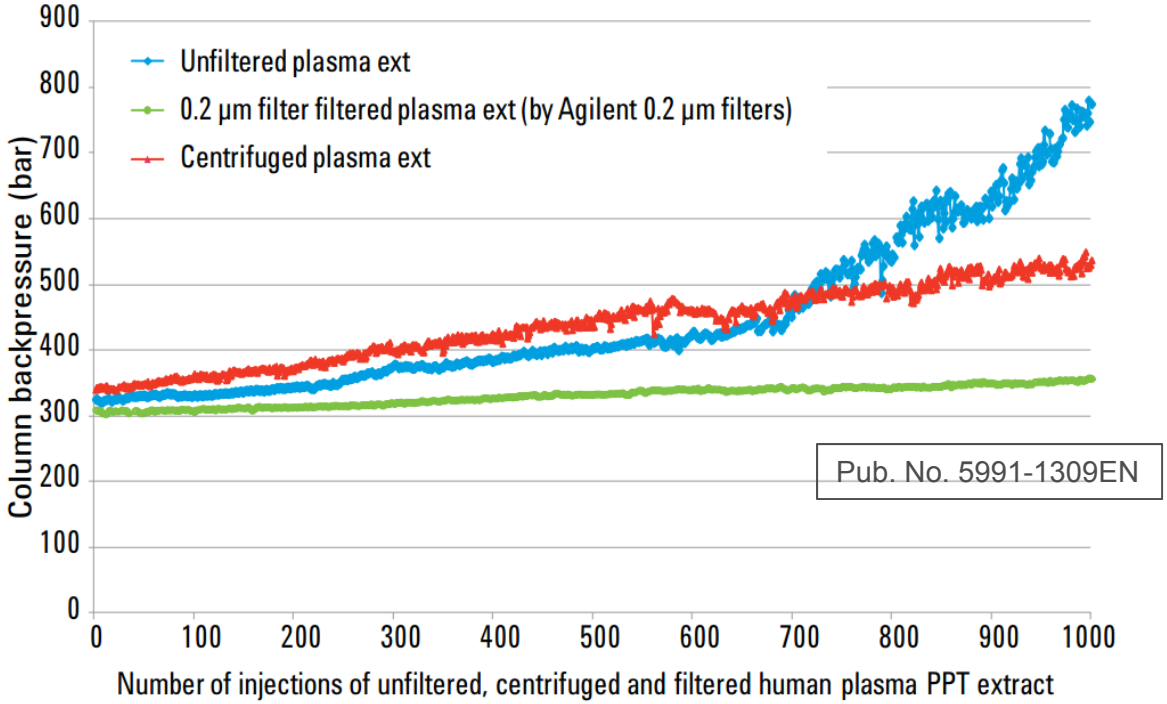
**Sample**: 10 µl injection,
5 µg peptide in 6M Urea/5% HOAc

**Conditions**: 1 ml/min
A=0.1%TFA in H2O, B=0.09%TFA in ACN
Gradient: 20-45% B / 35 min

- Changes k* and α*
- Potentially Improves Resolution ( R )
- **Know** if your sample is affected by temperature

# Sample

## Consider the effects of your sample matrix




Unfiltered, centrifuged, and filtered plasma extracts
Zorbax RRHD Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm column, PN 959757-902

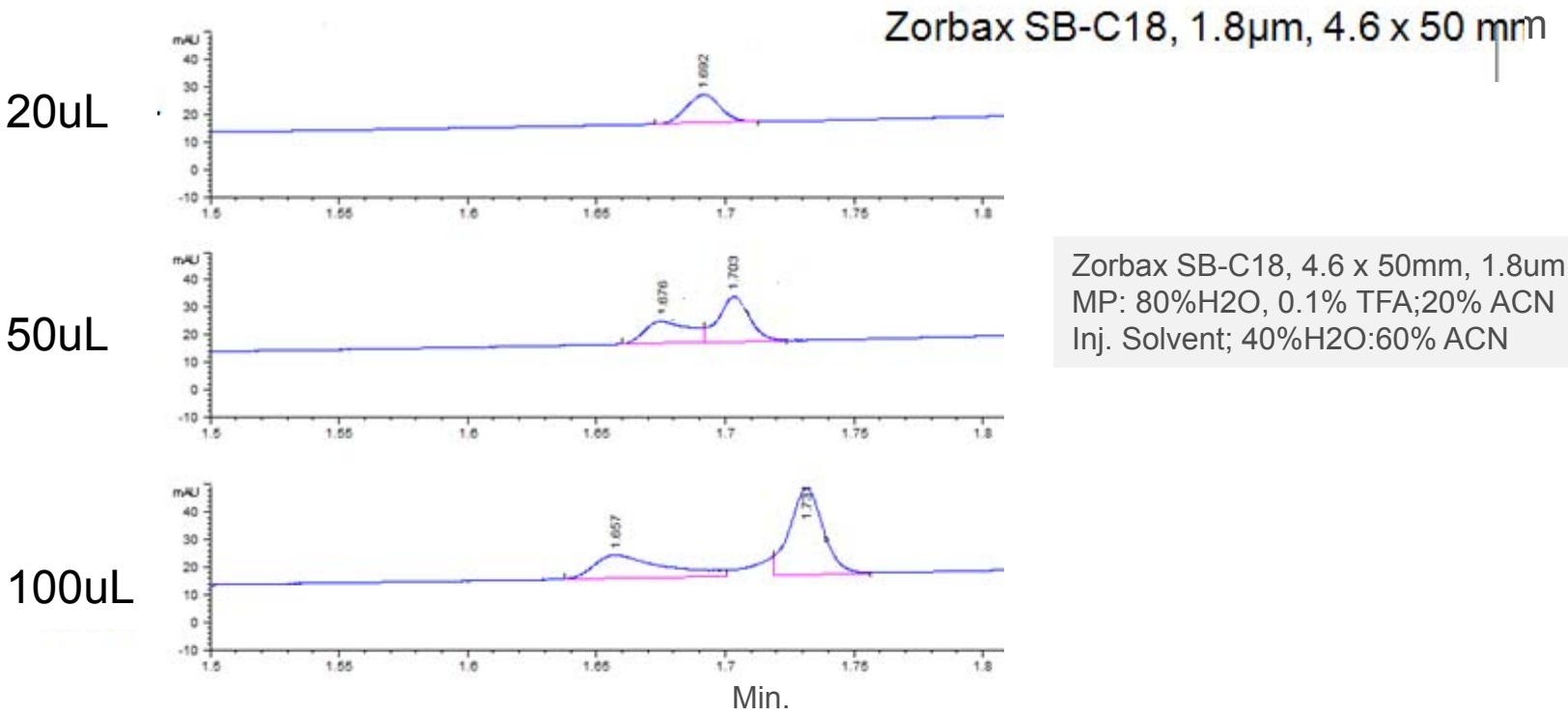
- Column plugging is one of the most common sources of LC column failure
- Especially with sub-2 µm columns, sample particulates can easily plug the column inlet frit

- To help avoid this pitfall, use an appropriate 0.2 µm filter with all samples prior to injection

http://filtrationselectiontool.chem.agilent.com

Captiva Filtration Selection Guide: 5991-1230EN

# Injection Solvent Effects




Zorbax SB-C18, 4.6 x 50mm, 1.8um
MP: 80%H2O, 0.1% TFA;20% ACN
Inj. Solvent; 40%H2O:60% ACN

➢Peak splitting is often observed when injecting a large volume of sample in a solvent that is stronger than the mobile phase

➢To avoid this pitfall

- When injecting a sample in strong solvent, limit the size of the injection
- Inject the sample in a solvent that is no stronger than the starting conditions for the method

# Conclusion

These are just a few of the potential pitfalls which we see fairly regularly.

Have you encountered other pitfalls or do you have an example of one we talked about today? If you have one you'd like to share for a future pitfalls talk, please send it to lc-column-support@agilent.com.

- Avoid Pitfalls -Check out the LC Handbook, Pub# 5990-7595EN

Thank you!

# Agilent Columns & Supplies Technical Support

## 800-227-9770 (Toll Free US & Canada)

- ***For LC columns***
- *Select option 3, then option 3, option 2*

*lc-column-support@agilent.com*

- ***For GC Columns***

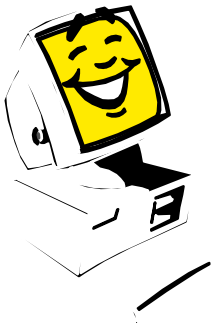
*Select option 3, then option 3, option 1*

*gc-column-support@agilent.com*

- ***For Sample Prep***

*Select option 3, then option 3, option 3*

- spp-support@agilent.com



- ***For Spectroscopy Supplies***

*Select option 3, then option 3, option 4*

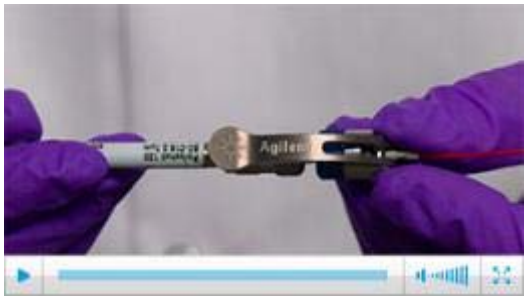
- spectro-supplies-support@agilent.com

www.agilent.com/chem

# Appendix

# Information about InfinityLab (A-line) Fittings

Video – how to use the A-Line fittings



Landing page:
www.agilent.com/chem/A-Line

Overview flyer (5991-5164EN)

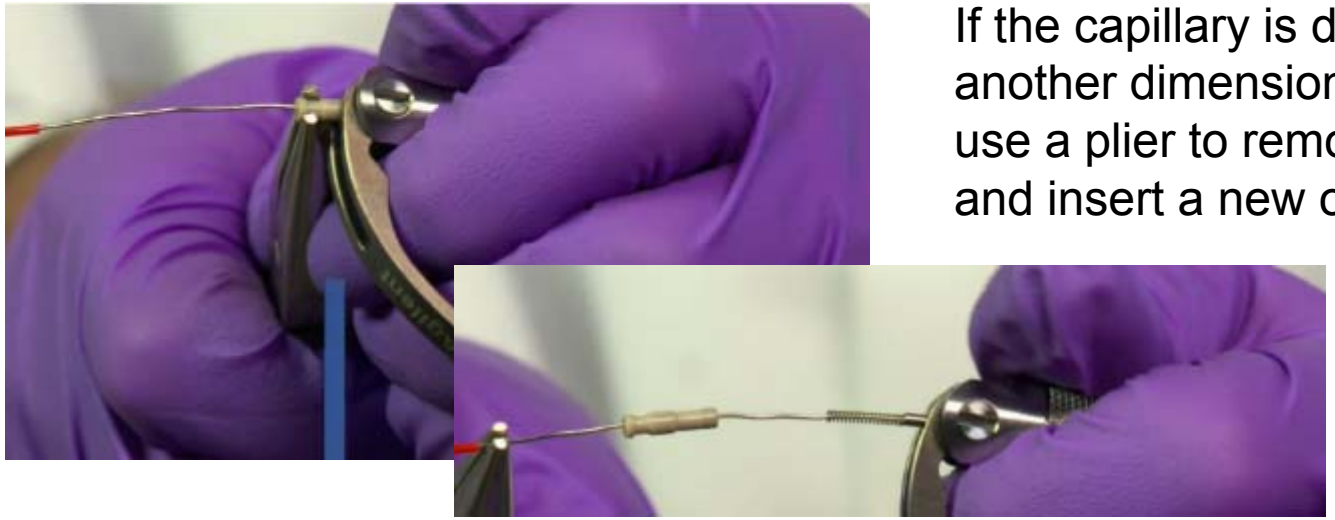
http://www.agilent.com/cs/library/brochures/5991-5164EN%20A-Line_Flyer.pdf


InfinityLab (A-Line) Fittings
Technical Overview

http://www.agilent.com/cs/library/technicaloverviews/public/5991-5525EN.pdf

Includes ordering info for the fitting, capillaries, and ferrules. Kits are available for easy ordering.

# Replacing capillaries and ferrules



If the capillary is damaged, or another dimension is needed, just use a plier to remove the capillary and insert a new one.

The ferrules can also be replaced; just use pliers to remove the ferrule and insert a new one.

# Column Cleaning

## Flush with stronger solvents than your mobile phase.

Reversed-Phase Solvent Choices
in Order of Increasing Strength

Use at least 25 mL of each solvent for analytical columns

- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile
- 75% Acetonitrile:25% Isopropanol
- 100% Isopropanol
- 100% Methylene Chloride*
- 100% Hexane*

* When using either Hexane or Methylene Chloride the column must be flu with Isopropanol before returning to your reversed-phase mobile phase.

# Separation Ruggedness
# Buffer Preparation

1. Dissolve salt in organic-free water in 1- or 2-L beaker. Use appropriate volume to leave room for pH adjustment solution. Equilibrate solution to room temperature for maximum accuracy.
2. Calibrate pH meter. Use 2-level calibration and bracket desired pH. Use appropriate audit solution to monitor statistical control (for example, potassium hydrogen tartrate, saturated solution, pH = 3.56).
3. Adjust salt solution to desired pH. Minimize amount of time electrode spends in buffer solution (contamination). Avoid overshoot and readjustment (ionic strength differences can arise).
4. Transfer pH-adjusted buffer solution quantitatively to volumetric flask, dilute to volume, and mix.
5. Filter through 0.45 µm filter. Discard first 50 – 100 mL filtrate. Rinse solvent reservoir with small volume of filtrate and discard. Fill reservoir with remaining filtrate or prepare premix with organic modifier.
   - Agilent Solvent Filtration Kit, 250-mL reservoir, 1000-mL flask, p/n 3150-0577
   - Nylon filter membranes, 47 mm, 0.45 µm pore size, p/n 9301-0895

# Using Buffers Successfully
# Initial Column and System Equilibration

In an appropriate vessel, test highest % organic/buffer ratio to verify that buffer will not precipitate. With stirring, add organic to buffer first, not vice versa.

Equilibrate column with, in order:

- 100% organic modifier (if brand new)
- mobile phase minus buffer
- buffered mobile phase containing highest % organic modifier (gradient high end)
- buffered mobile phase containing lowest % organic modifier (gradient low end).

Inject standard or sample several times until RTs stable, or for gradient methods, precede former with 1 or 2 blank gradients.

# Using Buffers Successfully
# Shutdown State and Instrument Flushing

## Shutdown State

Next day use—using same buffers

- Pump mobile phase very slowly (for example, 0.01 – 0.1mL/min).

When flushing column or for longer term column storage

- Flush with 20/80 organic/water, then 80/20 organic/water or 100% organic.

## Instrument flushing

Replace column with capillary tubing.  Leave disconnected from detector.

Flush pumps with water, then connect capillary tubing to detector.

Inject water 2-3 times at maximum injection volume setting.

Flush all pumps with 100% organic for long term storage.

# Method Validation Kits

| Agilent ZORBAX Rapid Resolution High Definition (RRHD) Method Validation Kits | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Size (mm) | Particle Size (µm) | Eclipse Plus C18 | Eclipse Plus C8 | Eclipse XDB-C18 | Extend-C18 | Eclipse Plus Phenyl-Hexyl | Bonus-RP | SB-C18 | SB-C8 | SB-Phenyl | SB-Aq |
| 3.0 x 150 | 1.8 | 959759-302K | 959759-306K | 981759-302K | | | | 859700-302K | 859700-306K | | |
| 3.0 x 100 | 1.8 | 959758-302K | 959758-306K | 981758-302K | 758700-302K | 959758-312K | | 858700-302K | 858700-306K | 858700-312K | 858700-314K |
| 3.0 x 50 | 1.8 | 959757-302K | 959757-306K | 981757-302K | 757700-302K | 959757-312K | | 857700-302K | 857700-306K | 857700-312K | 857700-314K |
| 2.1 x 150 | 1.8 | 959759-902K | 959759-906K | 981759-902K | 759700-902K | 959759-912K | 859768-901K | 859700-902K | 859700-906K | 859700-912K | 859700-914K |
| 2.1 x 100 | 1.8 | 959758-902K | 959758-906K | 981758-902K | 758700-902K | 959758-912K | 858768-901K | 858700-902K | 858700-906K | 858700-912K | 858700-914K |
| 2.1 x 50 | 1.8 | 959757-902K | 959757-906K | 981757-902K | 757700-902K | 959757-912K | 857768-901K | 857700-902K | 857700-906K | 857700-912K | 857700-914K |

| Agilent ZORBAX Method Validation Kits | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Size (mm) | Particle Size (µm) | Eclipse Plus C18 | Eclipse Plus C8 | Eclipse XDB-C18 | Eclipse XDB-C8 | Extend-C18 | Eclipse Plus Phenyl-Hexyl | Bonus-RP | SB-Aq | SB-C18 | SB-C8 | SB-Phenyl |
| 4.6 x 250 | 5 | 959990-902K | 959990-906K | 990967-902K | 990967-906K | 770450-902K | 959990-912K | 880668-901K | 880975-914K | 880975-902K | 880975-906K | 880975-912K |
| 4.6 x 150 | 5 | 959993-902K | 959993-906K | 993967-902K | 993967-906K | 773450-902K | | 883668-901K | 883975-914K | 883975-902K | 883975-906K | 883975-912K |
| 3.0 x 150 | 5 | 959993-302K | | | | | | | | | | |
| 4.6 x 250 | 3.5 | | | | | | | | | 884950-567K | | |
| 4.6 x 150 | 3.5 | 959963-902K | 959963-906K | 963967-902K | 963967-906K | 763953-902K | 959963-912K | 863668-901K | 863953-914K | 863953-902K | 863953-906K | 863953-912K |
| 4.6 x 100 | 3.5 | 959961-902K | 959961-906K | 961967-902K | 961967-906K | 764953-902K | 959961-912K | 864668-901K | 861953-914K | 861953-902K | 861953-906K | 861953-912K |
| 4.6 x 50 | 3.5 | 959943-902K | 959943-906K | 935967-902K | 935967-906K | 735953-902K | 959943-912K | 835668-901K | 835975-914K | 835975-902K | 835975-906K | 835975-912K |
| 4.6 x 150 | 1.8 | 959994-902K | | | | | | | 829975-914K | 829975-902K | 829975-906K | 829975-912K |
| 4.6 x 100 | 1.8 | 959964-902K | 959964-906K | 928975-902K | 928975-906K | 728975-902K | 959964-912K | 828668-901K | 828975-914K | 828975-902K | 828975-906K | 828975-912K |
| 4.6 x 50 | 1.8 | 959941-902K | 959941-906K | 927975-902K | 927975-906K | 727975-902K | 959941-912K | 827668-901K | 827975-914K | 827975-902K | 827975-906K | 827975-912K |
| 3.0 x 100 | 1.8 | | | | 928975-306K | | | 828668-301K | | | | |
| 3.0 x 50 | 1.8 | | | | 927975-306K | | | 827668-301K | | | | |
| 2.1 x 100 | 1.8 | | | | 928700-906K | | | | | | | |
| 2.1 x 50 | 1.8 | | | | 927700-906K | | | | | | | |

| Agilent Poroshell 120 Method Validation Kits | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Size (mm) | Particle Size (µm) | EC-C18 | EC-C8 | Phenyl-Hexyl | SB-C18 | SB-C8 | SB-Aq | Bonus-RP |
| 4.6 x 150 | 2.7 | 693975-902K | 693975-906K | 693975-912K | 683975-902K | 683975-906K | 683975-914K | 693968-901K |
| 4.6 x 100 | 2.7 | 695975-902K | 695975-906K | 695975-912K | 685975-902K | 685975-906K | 685975-914K | 695968-901K |
| 4.6 x 50 | 2.7 | 699975-902K | 699975-906K | 699975-912K | 689975-902K | 689975-906K | 689975-914K | 699968-901K |
| 3.0 x 150 | 2.7 | 693975-302K | 693975-306K | 693975-312K | 683975-302K | 683975-306K | 683975-314K | 693968-301K |
| 3.0 x 100 | 2.7 | 695975-302K | 695975-306K | 695975-312K | 685975-302K | 685975-306K | 685975-314K | 695968-301K |
| 3.0 x 50 | 2.7 | 699975-302K | 699975-306K | 699975-312K | 689975-302K | 689975-306K | 689975-314K | 699968-301K |
| 2.1 x 150 | 2.7 | 693775-902K | 693775-906K | 693775-912K | 683775-902K | 683775-906K | 683775-914K | 693768-901K |
| 2.1 x 100 | 2.7 | 695775-902K | 695775-906K | 695775-912K | 685775-902K | 685775-906K | 685775-914K | 695768-901K |
| 2.1 x 50 | 2.7 | 699775-902K | 699775-906K | 699775-912K | 689775-902K | 689775-906K | 689775-914K | 699768-901K |

# Method Development Kits

| PN | MD Kits | Description |
|---|---|---|
| 5190-6160 | **P120, USP Method Dev Kit, 3.0x100mm** | Poroshell 120 EC-C18, EC-C8, EC-CN columns, 3.0 x 100 mm |
| 5190-6159 | **P120, USP Method Dev Kit, 4.6x100mm** | Poroshell 120 EC-C18, EC-C8, EC-CN columns, 4.6 x 100 mm |
| 5190-6155 | **P120, Selectivity Meth Dev, 2.1x50mm** | Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm |
| 5190-6156 | **P120, Selectivity Meth Dev, 4.6x50mm** | Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 4.6 x 50 mm |
| 5190-6157 | **P120, Aqueous Meth Dev Kit, 2.1x50mm** | Poroshell 120 Sb-Aq, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm |
| 5190-6158 | **P120, Aqueous Meth Dev Kit, 4.6x50mm** | Proshell 120 SB-Aq, Phenyl-Hexyl, and Bonus RP columns, 4.6 x 50 mm |
| 5190-6153 | **RRHD Eclipse Plus Meth Dev Kit, 2.1mm ID** | RRHD Eclipse Plus C18, Eclipse Plus C8, Eclipse Plus Phenyl-Hexyl, 2.1 x 50 mm columns |
| 5190-6154 | **RRHD Aqueous Method Dev Kit, 2.1mm ID** | RRHD SB-Aq, Bonus RP, and Eclipse Plus Phenyl-Hexyl columnc, 2.1 x 50 mm |
| 5190-6152 | **RRHD pH Method Dev Kit, 2.1mm ID** | RRHD StableBond SB-C18, Eclipse Pluse C18, and Extend-C18 column, 2.1 x 50 mm |
| 5190-6160 | **P120, USP Method Dev Kit, 3.0x100mm** | Poroshell 120 EC-C18, EC-C8, EC-CN columns, 3.0 x 100 mm |
| 5190-6159 | **P120, USP Method Dev Kit, 4.6x100mm** | Poroshell 120 EC-C18, EC-C8, EC-CN columns, 4.6 x 100 mm |
| 5190-6155 | **P120, Selectivity Meth Dev, 2.1x50mm** | Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm |
| 5190-6156 | **P120, Selectivity Meth Dev, 4.6x50mm** | Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 4.6 x 50 mm |
| 5190-6157 | **P120, Aqueous Meth Dev Kit, 2.1x50mm** | Poroshell 120 Sb-Aq, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm |